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THE UNIVERSITY OF ALBERTA

THE PURIFICATION AND CHARACTERIZATION OF KINESIN

BY

GRAHAM HARDCASTLE BROCKLEY

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF
MASTER OF SCIENCE
IN
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DEPARTMENT OF SURGERY

EDMONTON, ALBERTA
FALL, 1987

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THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE PURIFICATION AND CHARACTERIZATION OF KINESIN submitted by GRAHAM HARDCASTLE BROCKLEY in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

(Supervisor)

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Date: Sept. 4/1987

ABSTRACT

Following the AMP-PNP-based microtubule-affinity purification of kinesin from squid axoplasm, squid optic lobe and bovine brain (Vale et al., 1985c), researchers have isolated kinesin-like proteins (k.l.p.'s) from a variety of tissue (see Figure 2, page 18). Different techniques have been used to bind kinesin or k.l.p.'s to microtubules, including the use of non-hydrolzable ATP analogues (AMP-PNP; AMP-PCP; PPP?; ADP-VO4; Mg^{++} depletion in the presence of ATP?) and the induction of a rigor-like condition (ATP-depletion; Mg^{++} depletion in the presence of ATP?).

We compared EDTA, AMP-PNP and PPP-induced binding as three different methods of performing microtubule-affinity purification of bovine k.l.p.. We propose that both EDTA and PPP chelate Mg^{++} in high speed brain homogenate and prevent ATP from entering into the k.l.p. ATPase site, thereby inducing a rigor-like state of binding between microtubules and k.l.p.. The use of Mg^{++} depletion as a purification method has not appeared in the literature, although it has been mentioned as a means of promoting the strong association of kinesin with microtubules (Vale et al., 1986b). A purification technique using endogenous microtubules was also developed; this procedure is simpler than published k.l.p. purification methods. Experiments with homogenization buffers were performed.

Microtubules to be used in the affinity purification of k.l.p.s were purified using the procedure of Vallee (1982) and characterized with polyacrylamide gel electrophoresis, scanning electron microscopy, video-enhanced differential interference contrast microscopy and transmission electron microscopy.

Bovine brain k.l.p. was characterized for subunit molecular weight using polyacrylamide gel electrophoresis, and for total molecular weight using gel

filtration chromatography. Immunological identification using an immunoblot technique was performed with polyclonal Rabbit anti-Squid kinesin. Isoelectric point determination was partially completed, using isoelectric focusing and known characteristics of the protein.

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TABLE OF CONTENTS

Page

I. INTRODUCTION

A. Early Speculation and Evidence for Axonal Transport 1

B. First Direct Evidence for Slow Transport 2

C. First Evidence for Fast Anterograde and Retrograde Transport and Definition of Different Transport Components 3

D. Fast Transport - Relations to Organelles 5

E. Models for Transport 6

F. Cytoplasmic Structure in Axons 8

G. Characterizing Fast Axonal Transport 9

H. The Nature of Kinesin and Kinesin-Like Proteins 12

1. Isolation Procedures and Binding Conditions 13
2. Molecular Weights 17
3. ATPase Activity 21
4. Motility 23
5. Pharmacological Studies 28
6. Antigenicity 29
7. Electron Microscopy 30
8. Mechanics of Kinesin Activity 31
9. Other Possible Cytoplasmic Motors 34

II. MATERIALS AND METHODS

A. Microtubule Purification and Characterization 37

1. Introduction 37
2. Purification Technique 38

- a. Taxol 38
- b. Procedure 38
- 3. Characterization 41
  - a. Electron Microscopy - Thin Section Transmission Electron Microscopy (TEM) 41
  - b. Electron Microscopy - Negative Staining 42
  - c. Scanning Electron Microscopy 42
  - d. Video-Enhanced Differential Interference Contrast Microscopy 43
- B. K.L.P. Purification and Characterization 44
  - 1. Introduction 44
  - 2. Isolation Technique 44
    - a. Microtubule Affinity Purification of K.L.P. (from Vale et al., 1985c) 44
    - b. Variations on the Isolation Procedure of Vale et al., (1985c) 48
      - (i) Homogenization Buffer 48
      - (ii) Other Variables 50
  - 3. Fast Protein Liquid Chromatography (FPLC) 55
    - a. Introduction 55
    - b. Procedure 56
      - (i) Column Standardization 56
      - (ii) Buffers 57
      - (iii) After-Column Concentration 59
        - a) Centricon : Principle; Retention and Recovery; Procedure; Results 60
        - b) Freeze Drying 63
        - c) Glass Adsorption 63

4. Gel Electrophoresis 66
  a. Polyacrylamide Gel Electrophoresis (PAGE) 66
    (i) Introduction 66
    (ii) Gel Casting and Running 67
    (iii) Gel Staining 68
    (iv) Gel Scanning 68
  b. Non-Denaturing Discontinuous Gels 69
    (i) Introduction 69
    (ii) Gel Casting and Running 69
    (iii) Staining 70
    (iv) Results 70
  c. Isoelectric Focusing 70
5. Immunoblot 72
  a. Introduction 72
  b. Procedure 73
6. Motility Tests 75

III. RESULTS 77
  A. Microtubule Purification and Characterization 77
  B. K.L.P. Purification and Characterization 88
    1. Introduction 88
    2. Notation 89
    3. Results 90
      a. Run #1 - Experiments with homogenization buffers; isoelectric focusing 90
      b. Run #2 - EDTA vs AMP-PNP; elimination of wash step 98
      c. Run #3 - Dog brain 101

d. Run #4 - Use of PPP; extra wash; glass wool technique 104

e. Run #5 - Immunoblotting 5-S_6; pinpointing k.l.p. losses 107

f. Run #6 - Squid kinesin; ATP-containing buffer 115

g. Run #7 - Endogenous microtubules; immunoblotting 7-S_2 120

h. Run #8 - Immunoblotting FLPC eluant 125

IV. DISCUSSION 132

A. Microtubules 132

B. Isoelectric focusing 133

C. SDS-PAGE 134

D. Ion Exchange Columns 134

E. Gel Filtration 136

F. After-Column Concentration 137

G. Non-Denaturing Discontinuous Gels 138

H. Immunological Identification 139

I. Purification Techniques - Alternatives 141

1. S6 K.L.P. Concentration 141

2. Binding Agent Changes 143

3. Alternative tissues 146

J. Conclusion: Simplified K.L.P. Purification Technique 147

1. Homogenization Buffer 147

2. Microtubules 147

3. Washes 147

4. Binding Agents 148

5. S_1:S_6 Ratios 148

V. REFERENCES 149

LIST OF FIGURES

Figure		Page
1	Actin-Myosin and Microtubule-Dynein Interactions	14
2	Kinesin and Kinesin-Like Proteins : Molecular Weights	18
3	Proposed Kinesin-Microtubule Interactions (Hill, 1986)	32
4	Purification of Microtubules	39
5	Microtubule-Affinity Purification of K.L.P. : Procedure of Vale et al. (1985c) (*Variations from the procedure of Vale et al. are printed in italics*)	45
6	Microtubule-Affinity Purification of K.L.P. : Using Endogenous Microtubules *(Variations from Figure 5 are printed in italics)*	53
7	Superose 6 : Elution Volume vs log Molecular Weight	58
8	Superose 6 : Percent Deflection vs µg Protein	58
9	Reference Solutions for Spectrophotometer	61
10	Recovery From Treated and Untreated Centricon 10 Units	62
11	Schleicher and Schuell Microfilter Apparatus	65
12	Run #1 - Gel Scanning of Bovine Brain S_6	95
13	Run #5 - Superose 6 Gel Filtration of Bovine Brain S_6	113
14	Run #8 - Superose 6 Gel Filtration of Bovine Brain S_6	130

LIST OF PHOTOGRAPHIC PLATES

Plate	Description	Page
I	SDS Polyacrylamide Gel Electrophoresis of Purified Bovine Brain Microtubules	79
II	AVEC-Dic of Purified Bovine Brain Microtubules	81
III	Thin-Section Transmission Electron Microscopy of Purified Bovine Brain Microtubules	83
IV	Transmission Electron Microscopy of Negatively-Stained Purified Bovine Brain Microtubules	85
V	Scanning Electron Microscopy of Purified Bovine Brain Microtubules	87
VI	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #1	92
VII	Isoelectric Focusing of Bovine Brain S_6 - Run #1	97
VIII	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #2	100
IX	SDS Polyacrylamide Gel Electrophoresis of Dog Brain S_6 - Run #3	103
X	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #4	106
XI	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #5	109
XII	Immunoblotting of Bovine Brain S_6 - Run #5	111
XIII	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 and Squid Kinesin - Run #6	117
XIV	Immunoblotting of Squid Kinesin - Run #6	119
XV	SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #7	122
XVI	Immunoblotting of Bovine Brain S_6 and S_2 - Run #7	124
XVII	Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 - Run #8	127
XVIII	Immunoblotting of Bovine Brain S_6 and Superose-6 Purified Bovine K.L.P. - Run #8	131

LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

AMP-PNP - adenyl-5'-imidodiphosphate, a non-hydrolyzable analogue of ATP

ATP - adenosine triphosphate

AVEC-DIC - Allen Video-Enhanced Contrast - Differential Interference Contrast (method for increasing the resolution of light microscopy)

B.A. - Binding Agent

Bis - N, N'-Methylene bis-acrylamide

BSA - bovine serum albumin

CNS - Central Nervous System

DTT - dithiothreitol

EDTA - Ethylene diamine tetraacetate Acid - Disodium salt

EHNA - erythro-9-(3-[2-hydroxnonyl]) adenine

GTP - Guanosine triphosphate

HRP - horseradish peroxidase

IDPN - ß, ß' - imidodipropionitrile

k.l.p. - kinesin-like protein

MAP - microtubule-associated protein

NEM - N-ethyl-maleimide

NGF - nerve growth factor

PAGE - polyacrylamide gel electrophoresis

PPP - inorganic tripolyphosphate

S_1 - crude homogenate of tissue

S_2 - homogenate of tissue after high speed centrifugation

S_3 - homogenate of tissue after high speed centrifugation and removal of endogenous microtubules

SCa - slow component a

SCb - slow component b

SDS - sodium dodecyl sulphate

TEMED - N_1N, N', N'-tetra methyl-ethylenediamine

Treatment Buffer - (0.315 M Tris pH = 6.8, 10% SDS, 50% glycerol, 0.25 M DTT)

I. INTRODUCTION

A. EARLY SPECULATION AND EVIDENCE FOR AXONAL TRANSPORT

Although the fact of axonal transport has only recently been established, the notion of nerves as conduits for the flow of some influential substance is very old. Early civilized man (e.g. 6th century B.C. Greeks), drawing on earlier animistic traditions, believed an animal spirit (pneuma) was drawn into the body via respiration and thereafter served to animate the body and power sensory processes and, to some degree, thought. Galen (130-200 A.D.), believed that vital spirit produced via respiration was carried through arteries to "vivify" the body; the brain removed finer spirits from the blood and used these spirits to produce psychic functions, caused by movement of animal spirits within the ventricles, and muscular control, caused by the movement of CNS animal spirits down into the peripheral nervous system. The concept of animal spirits moving in hollow tubes remained influential into the nineteenth century (Ochs, 1982).

Van Leeuwenhoek's microscope enabled him to make crude observations of optic nerves (1717); further advances before the twentieth century roughly paralleled advances in microscopy. Fontana (1778) was able to see individual nerve fibers. The introduction of the achromatic compound lens in the early 1800's led to rapid advances. Purkinje and Valentin (Ochs, 1982) identified the cell body and differentiated between the nucleus and the nucleolus, but due to a lack of staining technique they concluded that cell bodies and nerve fibers were separate entities. They believed that the cell body was the "active" element which caused the "passive" nerve fluid contained in the continuously-looped nerve fibers to circulate. Waller's classic experiments in nerve degeneration (1852) established that some "trophic factor" from the cell body was indeed necessary to maintain fiber viability, but the nature of the relationship between these two elements remained hazy. Remack (1838) suggested and Ramon y Cajal (1894, 1909) established that the nerve fibers and cell bodies were in fact connected and formed a single entity, with the cell body considered to be the neuron's trophic center. The nature and mechanism of the nuclear control was unknown, but Ramon y Cajal (1909) suggested that "perhaps the neuroplasm, situated among the neurofibrils in the cell body and in the axon, serves as a diffusion pathway for a substance or substances still unknown, which have a trophic action and are elaborated by the nucleus." He observed that a severed axon will regenerate by forming a growth cone which then made its way toward the periphery, just as the original growth cone had done in the embryo (Ochs, 1982). F.H. Scott (1905), noting chemical and structural similarities between the two cell types, suggested that neurons were secretory cells that transported proteins or secretion particles to nerve terminals. This was an idea years ahead of its time.

The next major step was taken by Ross G. Harrison (1910) who invented tissue culture. He showed that axons could grow without the support of Schwann cells, which demonstrated that neither the growth cone nor the axon were formed by surrounding cells. This implied that materials must be supplied by the cell body to the axon during the extension of the axon; whether material was moving in non-growing axons was an issue that was debated but not resolved until after World War Two when the treatment of war injuries sparked new interest in the study of nerve regeneration.

B. FIRST DIRECT EVIDENCE FOR SLOW TRANSPORT

Weiss and Hiscoe (1948) tried to determine whether radial axonal growth was due to *de novo* axoplasmic synthesis or due to transport of components synthesized in the cell body. Rat tibial or peroneal nerve was crushed to trigger regeneration and constricted distal to the crush. Progressive swelling was seen proximal to the constriction; Bodian staining showed this to be an accumulation of axoplasm. After four weeks, the constriction was released and the axoplasmic swelling moved down the axon at 1-2 mm/day. They postulated that this represented the normal rate of axoplasmic flow. Subsequent re-examination of these results (Spencer, 1972) suggested that the effects described by Weiss do not occur in normal axons. However, this was the first direct evidence of slow axonal transport.

Weiss and Hiscoe (1948) also noted that when a polio virus was inoculated into the periphery it made its way retrogradely, against the bulk anterograde flow, from the nerve terminals up into the alpha motor neuron cell bodies at a velocity of several mm/day. This early evidence for retrograde transport was considered to be possibly artifactual; the existence of retrograde transport did not become generally accepted until Lubinska (1964) suggested that in addition to slow transport, rapid movement of intra-axonal particles both away from and toward the cell body was occurring.

C. FIRST EVIDENCE FOR FAST ANTEROGRADE AND RETROGRADE TRANSPORT AND DEFINITION OF DIFFERENT TRANSPORT COMPONENTS

Lubinska (1964) based her conclusions on the following experiment: Canine sciatic nerve was ligated in two places to isolate a segment 5 cm long. The nerve was removed 2 hours to several days later and segments were measured for acetylcholinesterase activity. Accumulation of activity was found both proximal and distal to each ligature. This result led to three conclusions: 1) This enzyme moves in a retrograde as well as an anterograde direction; 2) The transport mechanism functions at all points in the axon, since enzyme accumulation occurred within the isolated segment; 3) Accumulation was detected within two hours of ligation; therefore, both anterograde and retrograde transport were occurring at rates much faster than previously detected. Subsequently, Dahlstrom (1965) used fluorescense to observe the accumulation, within 45 minutes, of catecholamines proximal and distal to ligatures placed on the greater splanchnic nerves of rats.

Radioactively labelled proteins were used to examine fast and slow transport. Droz and Leblond (1963) systemically injected radioactive amino acids that first accumulated in neuronal cells and later could be detected as a wave of radioactive protein moving down the axon at a rate of 1 mm/day. High background radiation in glial cells prevented the detection of a fast component. Between 1966 and 1968, by using more specific application techniques, investigators were able to observe radioactive material moving within axons at faster rates. Lasek (1968) injected cat L-7 dorsal root ganglia with radioactive leucine and removed the sciatic nerve and branches between 14 hours and 60 days after the injection. The nerve was divided into 5 mm segments and radioactivity was measured. He concluded that two transport velocities were present, one fast (100-500 mm/day) and one slow (1-2 mm/day) (see also Grafstein, 1967).

Subcellular fractionation allowed the separation of radiolabelled fast and slow transport components (McEwan and Grafstein, 1968; Lorenz and Willard, 1978). Fast transport was shown to be associated with plasma membrane and particulate fractions; materials transported included neurotransmitters, enzymes for neurotransmitter metabolism (Goldman et al., 1976) and glycoproteins (Ambron et al., 1974). Slow transport was shown to be associated with fibrous cytoskeletal components and soluble proteins; further work (Black and Lasek, 1980) identified two subcomponents of slow transport:

SCa moves at 1mm/day and makes up the neurofilament and microtubule network, while SCb moves at 0.25 mm/day and is composed of a complex of 50 or more proteins (including "soluble" proteins, enzymes, actin, myosin, fodrin, nerve specific enolase, creatine phosphokinase, aldolase, plus others - see Black et al., 1979; Brady and Lasek, 1981; Levine and Willard, 1981; Willard and Hulebak, 1977).

D. FAST TRANSPORT - RELATION TO ORGANELLES

Kristensson and Olsson (1971) applied horseradish peroxidase (HRP) to the gastrocnemeus muscle in mouse. HRP reaction product was subsequently found within intra-axonal tubular structures and vesicular organelles and, after 24 hours, within the ipsilateral spinal motoneurons. This technique not only provided evidence for the localizing of retrograde transport within organelles, but also was widely used for tracing neuroanatomical pathways. Later work with nerve growth factor (Hendry et al., 1974; Stockel et al., 1975) and other proteins (Schwab et al., 1977, 1979, 1983; Purves and Lichtman, 1978) showed that different populations of neurons have a variety of receptor-mediated mechanisms for the uptake and retrograde transport of specific ligands (NGF - adrenergic neurons and sensory neurons; tetanus toxin and WGA - all neurons). HRP is transported non-specifically (Stockel et al., 1974).

These retrogradely transported proteins were contained within vesicular compartments. No similar label was available for anterograde transport; autoradiography coupled with electron microscopy did not have sufficient resolution to identify single organelles (only bulk movement of organelles can be seen with autoradiography). Direct association of fast anterograde transport with vesicles was provided by light microscopy of living axons (Cooper and Smith, 1974; Foreman et al., 1977). Particles could be seen moving at velocities consistent with rapid transport in both the retrograde and anterograde directions. However, 90% of the detectable particles were moving in the retrograde direction. This was later explained by two different papers in which investigators described the use of compression (Smith, 1980) or low temperature (Tsukita and Ishikawa, 1980) to block axonal transport. Organelles accumulating on the proximal and distal sides of the block were analyzed by electron microscopy. Retrogradely moving organelles (distal) were larger multi-lamellated vesicles (0.1-0.5 µm) and hence easier to detect with light microscopy than anterogradely moving vesicular-tubular bodies (0.04- 0.1 µm).

Further information was provided by the application of video processing to polorization-based microscopy (Allen et al., 1981; Inoué, 1981). Amplification of image contrast allowed structures below the resolution limit of optics (about 0.2 µm) to be detected; studies of the squid giant axon showed organelles less than 200 nm in diameter moving in both directions parallel to linear elements. Most of the very small organelles were moving anterogradely. In contrast to the saltatory movements of larger organelles, these small organelles moved continuously at 2.5 µm/sec, a rate consistent with fast axonal transport (Allen et al., 1982). These organelle movements continue in axoplasm that is extruded from the squid giant axon (Brady et al., 1982).

E. MODELS FOR TRANSPORT

Many different models for rapid vesicle transport have been put forward. Schmitt (1968) prophesied that all fast-transported materials were vesicles with surface projections that could temporarily bind to complementary sites of the microtubule; he envisioned the vesicles rolling down the microtubules using ATPase power for movement. Huxley's (1969) sliding filament theory of skeletal muscle contraction prompted Ochs (1971, 1972) to offer the "transport filament" model of fast axonal transport. In this model the common carrier, the transport filament, binds the various materials to be transported. The filaments are moved along the microtubules, the stationary element, by means of side-arms projecting from the microtubules in a process similar to muscle contraction. The failure to identify possible transport filaments and other practical problems caused this model to be eventually discarded.

The microstream hypothesis (Gross, 1975) stated that fast transport of materials takes place within low-viscosity channels surrounding the microtubules, with ATP supplying the energy. This hypothesis was still considered a possibility in 1985 (Schnapp et al., 1985). Weiss (1970) supported the microstream hypothesis, but also proposed an alternate mechanism based upon a "peristaltic" movement coursing down microtubules. Energy originating at one end would cause a rippling conformational change to be serially passed down the length of the microtubule. No experimental evidence was ever found for this model. Sheetz et al. (1984) proposed that vesicles contained a proton pump which provided a direct method of propulsion. This position was later abandoned by the group in light of new experimental evidence. Finally, the unitary hypothesis held that slow transport could be accounted for as an aspect of the same mechanism powering fast transport; the slow transport was simply due to the drop-off of certain classes of materials from the transport filaments (Ochs, 1975). This theory ran into difficulties when it was pointed out that the peak of radioactivity seen accompanying slow transport did not broaden or diffuse, but remained sharp (Ochs, 1982). Grafstein and Forman emphasized two theories in their formidable review published in 1980: the microstream hypothesis, and the hypothesis that a translocator protein acted directly against stationary filamentous structures (Grafstein and Forman, 1980).

F. CYTOPLASMIC STRUCTURE IN AXONS

In an attempt to expose the underlying mechanism of axonal transport, Schnapp and Reese (1982) rapidly froze living turtle optic nerves. Three-dimensional views of the insides of axons were obtained by fracturing the axons, micro-etching the intracellular water to avoid structural collapse, rotary replicating with platinum, and viewing via electron microscopy. This showed that the axonal cytoplasm was compartmentalized into three types of longitudinally oriented domains: neurofilament domains, which contain individual filaments interconnected by a cross-bridging network; microtubule domains, which contain microtubules suspended in a loose, granular matrix (possible complexes of soluble proteins comprising SCb); and a third domain, present only in an 80-100 nm wide zone next to the axonal membrane, which consists of a dense filamentous network that may connect the axoskeleton to the axolemma. Mitochondria and vesicular organelles are present only in the microtubule domains; Schnapp and Reese proposed that the longitudinally oriented microtubule domains are channels within which organelles are transported. They also showed that mitochondria are always attached to one or more microtubules by side arms, whereas vesicular organelles are not.

Microtubule domains form longitudinal channels; IDPN, a neurotoxin, can be used to collapse all the microtubule domains into the center of the axon, forming one large microtubule domain surrounded by neurofilament-associated cytoplasm (Griffin et al., 1983). All vesicular organelles were contained within this central region, and rapid transport occurred exclusively within this region. This supported the view of Schnapp and Reese that rapid vesicular transport was associated with microtubule domains, but failed to expose the underlying mechanism.

G. CHARACTERIZING FAST AXONAL TRANSPORT

Adams (1982) used high voltage discharges to puncture holes in the plasma membrane of giant axons from the legs of the crab *Carcinus maenas.* Under a light microscope, fast axonal transport could be seen to decrease as metabolites leaked out of the axon; movement could be reactivated by the addition of exogenous ATP, thus showing directly for the first time that rapid motion in axons was an ATP-requiring process.

The next major step was taken in 1983 by Hayden and collegues, who presented immunocytochemical evidence that transport filaments seen to be parallel to rapid vesicle transport were indeed microtubules (Hayden et al., 1983). Indirect immunofluorescence using a monoclonal antibody against alpha-tubulin showed that all transport filaments contain alpha-tubulin. Subsequent work with fluorescent phalloidin, a plant toxin that binds to filamentous actin, showed that transport filaments do not contain actin (Schnapp et al., 1984).

The next series of observations revealed that the transport filaments could in fact be single microtubules. In October of 1984, at the fourteenth annual meeting of The Society for Neuroscience, T.S. Reese presented immunofluorescence, AVEC-DIC, and electron microscopic evidence that a single microtubule could support transport; this work was published in *Cell* in February 1985 (Schnapp et al.,1985). When the axoplasm of the squid giant axon was placed in a small volume of ATP-containing buffer, large numbers of apparently single axoplasmic filaments dissociated completely from the bulk of the axoplasm. (Vale et al., 1985a). As perceived with AVEC-DIC microscopy, all organelles, regardless of size, moved continuously along isolated transport filaments at 2.2 µm/sec as long as ATP was present. In intact axoplasm, movements of the larger organelles was slow and saltatory, implying that the dense network of surrounding axoplasmic components impeded passage. Organelles attached to and moved along more than one filament at a time, suggesting that organelles had multiple binding sites for the unidentified motor. At places where filaments crossed one another, organelles could switch filaments. No movement occurred in the absence of ATP with 1 mM sodium azide and 10 mM 2-deoxyglucose present to block endogenous production of ATP. Under these conditions, organelles bound to filaments without moving. This state was compared by the experimenters to the rigor complex that develops in muscle or in cilia when ATP is absent.

In the same issue of *Cell*, the same group of investigators further detailed their observations of movement on single microtubules (Schnapp et al., 1985). The same dissociated single filaments viewed by AVEC-DIC not only transported organelles bidirectionally, that is, both anterogradely and retrogradely, but also allowed organelles moving in the same or opposite directions to pass each other without colliding. This indicated that each transport filament had several tracks for organelle movement. Characterization by electron microscopy of microfilaments shown by the video microscope to support movement revealed structures 22-27 nm in diameter, approximately ten times smaller than their apparent diameter measured from video micrographs, with substructures indicative of single microtubules.

Further information was supplied by Gilbert and Sloboda who isolated axonal vesicles from the squid giant axon and labeled them with rhodamine (Gilbert and Sloboda, 1984). They observed bidirectional ATP dependent transport of these fluorescent vesicles. In experiments in which proteins on the surface of the fluorescent vesicles were digested with trypsin before injection, no movement of these vesicles was seen, although transport of endogenous vesicles and organelles proceeded normally. It seemed that this motor was either attached to the vesicular membrane or was dependent for its action upon a protein in the vesicular membrane, possibly a binding protein.

Vale et al., (1985b) next showed that a soluble protein, present in axoplasmic supernatant, was necessary for movement to occur. They developed a reconstituted system composed of three components: MAP (microtubule-associated protein) free microtubules, vesicles, and axoplasmic supernatant. This axoplasmic supernatant was prepared by homogenizing squid axoplasm and preparing a high speed supernatant devoid of microtubules and organelles. A three-step sucrose gradient was used to separate microtubules, organelles, and a supernatant (S2) containing all the soluble components of the axoplasm. An aliquot of the organelle fraction was combined with MAP-free squid optic lobe microtubules and 2 mM ATP in the presence or absence of S2. The number of organelles moving along the microtubules in the presence of S2 was markedly increased. The velocity of such movement was the same as in dissociated axoplasm. The axoplasmic S2 also supported movement of carboxylated latex beads along microtubules or of microtubules on glass. The direction of microtubule movement on glass was opposite to that of organelle movement on microtubules. The factor in the S2 supernatant that promoted movement was sensitive to heat, trypsin, 100 µM vanadate, and AMP-PNP (adenyl-5'-imidodiphosphate, a non-hydrolyzable analogue of ATP). Microtubules did not move on poly-D-lysine coated coverslips in the presence of S2 supernatant.

Lasek and Brady (1985) were the first to note that AMP-PNP inhibited vesicle transport, and that axoplasmic vesicles form stable complexes with microtubules in the presence of AMP-PNP. These observations were used by Vale et al. (1985c) to partially purify translocator protein from S2 supernatant. The purification depended upon the translocator protein's unusual property of forming a high-affinity complex with microtubules in the presence of AMP-PNP. This protein, which they labeled "kinesin", migrated on gel filtration columns with an apparent molecular weight of 600 kilodaltons (kd). SDS-polyacrylamide gel electrophoresis (PAGE) analysis revealed subunit polypeptides of 110-120 and 60-70 kd. This protein was distinct in molecular weight and enzymic behavior from myosin or dynein. How kinesin was exerting force to generate movement remained unclear, as did the question of how both anterograde and retrograde transport could be seen on a single microtubule of fixed polarity. Despite such remaining questions, the publication of this report was a pivotal point in the study of axonal transport and intracellular motility. The implications of this new mechanochemical protein were evident to all, and consequently many groups have since used similar techniques to isolate kinesin-like proteins (k.l.p.'s) from other sources.

H. THE NATURE OF KINESIN AND KINESIN-LIKE PROTEINS

1. ISOLATION PROCEDURES AND BINDING CONDITIONS

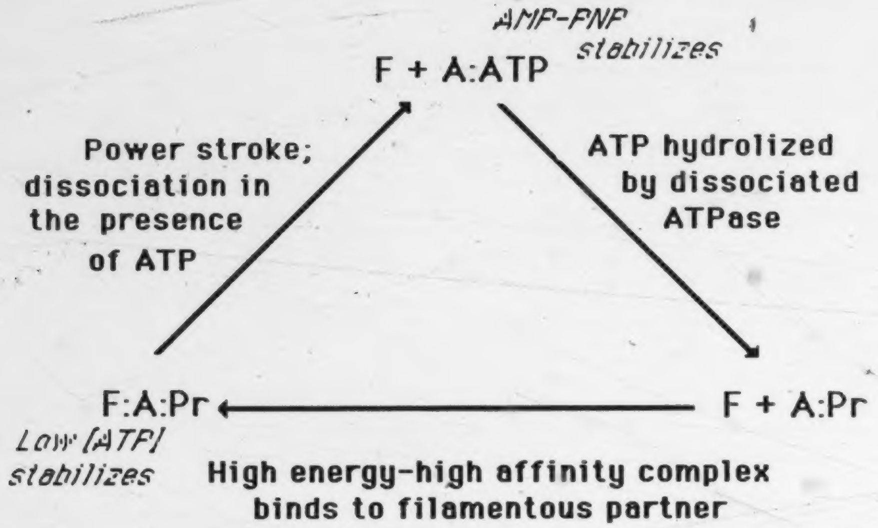
There have been a variety of procedures used to isolate k.l.p.'s from various tissues. An examination of these procedures will allow statements to be made about the mechanism of kinesin's action.

The original purification method by Reese's group was based upon Lasek and Brady's (1985) discovery that AMP-PNP causes organelles to bind to microtubules. This technique was used to isolate a k.l.p. from chick brain (Brady, 1985) and also from cow brain (Wagner et al., 1986). AMP-PNP has also been used to isolate k.l.p.'s from rabbit kidney and liver (Vale et al., 1986a), DU 145 human tumor cells (Piazza and Stearns, 1986), pig brain (Amos, 1987), sea urchin eggs (Scholey et al., 1985), and *Drosophila* embryos, adult heads, adult bodies, and cultured cells (Saxton et al., 1986). The implications of AMP-PNP binding were first explored by Lasek and Brady (1985), who noted that the effects of AMP-PNP on the vesicle transport system indicated that the enzymatic machinery of this system differed significantly from that of the actomyosin or the dynein-microtubule system.

The known cycle for both actomyosin and dynein-microtubule interactions begins with the binding of ATP to the stable actomyosin or dynein-ß subfibril complex ("rigor" complex) which induces a rapid release of the myosin/dynein crossbridge from its filamentous partner (see Figure 1). When no ATP is available, the rigor complex is maintained. ATP is hydrolyzed by the dissociated ATPase; this energy is stored by the formation of an ADP-(myosin/dynein) complex with a high affinity for the filamentous partner. The complex binds to its partner, releases its stored energy by going through a conformational change (the power stroke), and then dissociates from the filamentous protein when more ATP is available. AMP-PNP, a non-hydrolyzable analogue of ATP, weakly inhibits this ATPase cyle and, like ATP, promotes disassembly of the myosin-actin and dynein-microtubule complexes. This contrasts with the stabilization of the kinesin-microtubule complex seen with AMP-PNP (as attested to by the action of AMP-PNP on vesicle transport and by the possibility of using AMP-PNP in the isolation of kinesin). Therefore it seems there is a fundamental difference in the ATPase cycle of kinesin as compared to the other two known mechanochemical ATPases.

ATP depletion in actomyosin or dynein-microtubule systems causes a rigor state. The action of ATP-depletion on kinesin-microtubule systems is more

Figure 1: Actin-Myosin and Microtubule-Dynein Interactions


AMP-PNP stabilizes
F + A:ATP
Power stroke; dissociation in the presence of ATP
ATP hydrolized by dissociated ATPase
F:A:Pr
F + A:Pr
Low [ATP] stabilizes
High energy-high affinity complex binds to filamentous partner

F= filamentous partner (actin or microtubule)

A= ATPase (myosin or dynein)

Pr= products

uncertain. Lasek et al., (1985) reported that when ATP was eliminated from squid axoplasm by the enzyme apyrase, transport was inhibited and organelles exhibited Brownian motion, implying that no rigor complexes are formed but that ATP is necessary for transport to occur. However, Vale et al., (1985a) found that in dissociated squid axoplasm, absence of ATP caused the binding of organelles to filaments; they compared this state to myosin / actin or dynein / microtubule rigor complexes. Subsequently, Vale et al., (1985c) reported that kinesin would cosediment with microtubules in the absence of AMP-PNP if the S2 supernatant was first depleted of ATP using hexokinase and glucose. Scholey et al., (1985) also cosedimented a k.l.p. with microtubules by using apyrase to deplete the ATP concentration in sea urchin egg extract. Lately, Vale et al., (1986b) have reported that kinesin will bind strongly to microtubules when ATP is depleted.

The following conditions also seem to promote strong association of k.l.p.s with microtubules: (1) presence of inorganic tripolyphosphate (PPP) (Kuznetsov and Gefland, 1986); (2) presence of 1 mM ADP + 100 μM sodium vanadate (Scholey et al., 1985); (3) depletion of Mg^{++} in the presence of ATP (Vale et al., 1986b); (4) presence of AMP-PCP, which is less effective than AMP-PNP in causing k.l.p. binding to microtubules (Vale et al., 1986b). Both PPP and EDTA will inhibit axonal transport of vesicles (R.S. Smith, personal communication). Whether all the known ways of inducing k.l.p.-microtubule association are dependent upon the same binding mechanism seems doubtful; it is likely that at least two types of stabilized complexes are involved, one dependent upon a rigor-like condition (ATP depletion; Mg^{++} depletion?) and one dependent upon the intercalation of a non-hydrolyzable analogue of ATP (AMP-PNP; AMP-PCP; PPP?; ADP-VO4; Mg^{++} depletion?). This is supported by recent work by Vale et al. (1986b) which shows that the concentrations of MgATP necessary to release kinesin from AMP-PNP-induced attachment (1/2 max. at 2 mM) compared to ATP depletion (1/2 max. at 75 μM) are different.

Other recent work by Schnapp et al., (1986) studied kinesin-induced microtubule movement in a cell designed for rapid solution replacement. Sliding of microtubules stopped abruptly during a 10 second exchange in either 0 ATP, 0 Mg^{++}, or 10 μM AMP-PNP. Microtubules whose movement was stopped by 0 ATP or 0 Mg^{++} regained activity within 10 seconds after flooding with 1mM ATP + 5 mM Mg^{++}; recovery from AMP-PNP took over one minute. Washing the AMP-PNP-stalled microtubules with 0 Mg^{++} 0 nucleotide buffer for over 15 min did not reduce the one minute latency period when MgATP was introduced; however if the AMP-PNP-stalled microtubules were washed in a solution containing 1mM ATP (no Mg^{++}), the one minute latancy period was reduced to 10 seconds. They interpret these results as follows: since 0 Mg^{++} 0 nucleotide buffer will not reduce the AMP-PNP latancy period, this latency period is not due to a simple passive slow release or diffusion of AMP-PNP into solution. Adding ATP to this wash buffer, though, does eliminate the latency period; possibly the ATP is replacing the AMP-PNP at the active site of kinesin or, as they hypothesize, possibly ATP is attaching to a second active site, each functional unit of kinesin being considered to consist of two equivalent monomers. This second binding would cause the release of AMP-PNP (occupation of both sites by ligands is considered to be energetically unfavorable) but the lack of Mg^{++} prevents movement. They further hypothesize that release of hydrolysis products at one site could be driven by ATP binding at the other site; alternating activity at these two sites would reduce the chances of kinesin detatching from the microtubule during the mechanochemical cycle, during which they suggest there is antagonistic binding for kinesin by the microtubule and ATP.

Vale et al., (1986b) have also shown that dissociation of kinesin from microtubules is promoted by ATP, ADP,or ATP(gamma)s, which suggests that ATP hydrolysis is not required for kinesin's release. Mg^{++} (Ca^{++} will not substitute) is also required along with the nucleotide in order for kinesin release to occur. This lends authority to their hypothesis that nucleotide binding alone is sufficient to promote dissociation; whether or not this occurs at a second site remains to be proven.

To summarize, the stabilization of kinesin-microtubule association by AMP-PNP implies a different ATPase cycle is at work compared to actomyosin or dynein-microtubule interactions. There are a variety of other agents and conditions which will also promote this stabilization; these may hypothetically be divided into two types, one dependent upon a rigor-like condition and one dependent upon the intercalation of a non-hydrolyzable analogue of ATP. Recovery of microtubule motion on glass after stalling microtubules with AMP-PNP takes time; the limiting step seems to be the release of AMP-PNP from kinesin. This is not a passive process, but may be accelerated by the presence of ATP (which may be interpreted as supporting a "two active site" structure for kinesin).

2. MOLECULAR WEIGHTS

It may be premature to gather all these "k.l.p.'s" in one group. There are many variables which confuse the issue, such as molecular weight (see Figure 2). An AMP-PNP technique has been used by two investigators to isolate kinesin from cow brain. Vale et al., (1985c) initially reported bovine kinesin as a 600 kd protein which was composed of 120 and 60 kd subunits in

Figure 2: Kinesin and Kinesin-like Proteins : Molecular Weights

	Protein Size	Major Subunit	Other Subunits
Bovine Brain			
Vale et al.,1985c	600 KD	120 kd	60 kd (120:60 = 2:1)
Wagner et al.,1986	400	124	-
Kuznetsov and Gefland, 1986	'large'	135(55%)	66(21%), 70(11%), 58(10%), 45 (5%) (% = of total-by wt.)
Porcine Brain			
Amos, 1987	n/a	120	63
Sea Urchin Egg			
Scholey et al.,1985	500	134	-
Chick Brain			
Brady, 1985	n/a	130	-
Drosophila			
Saxton et al., 1986	n/a	115	-
DU 145 Human Tumor Cells			
Piazza et al., 1986	n/a	120	-
Squid Axoplasmic			
Vale et al.,1985c	600	110	-
Squid Optic Lobe			
Vale et al.,1985c	600	110	80 (110:80 = 4-10:1) 65/70(110:65/70 =1.5-2.2:1)
	(see text)	proteolytic fragments	60, 25 + 40 kd fragments (still bind to microtubules with AMP-PNP)
	(see text)	kinesin bound to microtubules, then exposed to ATP (0 Mg)	80 kd protein released
	(see text)	kinesin-binding proteins exist which show AMP-PNP-dependent binding to microtubules	

approximately a 2:1 ratio. Wagner et al., (1986) subsequently reported the isolation of a 400 kd bovine k.l.p., probably composed of three 124 kd subunits. Both groups used identical binding techniques; the reason for the discrepancy in their results is not clear. Kuznetsov and Gefland (1986) also isolated a "large" protein from cow brain using PPP which gave subunits of 135 (55% total protein composition), 66 (21%), 70 (11%), 58 (10%), and 45 (5%) kd. They identified this protein as bovine kinesin at least partially because it had "similar polypeptide composition" to bovine kinesin isolated by Vale et al., (1985c).

K.l.p.'s isolated from other tissues also exhibit a range of sizes: pig brain k.l.p., isolated using hexokinase + glucose + 0.5 mM AMP-PNP, has 120 kd and 63 kd subunits (Amos, 1987); sea urchin egg k.l.p., isolated using AMP-PNP, 1mM ADP + 100 μM vanadate, or ATP depletion with apyrase, has a molecular weight of 500 kd, composed of multiple 9.5 S subunits of 134 kd (Scholey et al., 1985, 1986); chick brain k.l.p., isolated using AMP-PNP, is composed of 130 kd subunits; *Drosophila* k.l.p., isolated using AMP-PNP, is composed of 115 kd subunits (Saxton et al., 1986); DU 145 human tumor cell k.l.p., isolated using AMP-PNP, is composed of 120 kd subunits; squid axoplasmic kinesin, isolated using AMP-PNP, is composed of 110 kd subunits, although squid optic lobe kinesin, isolated using AMP-PNP, is composed of 110, 80, and 65/70 kd subunits in a ratio of 1.5:1-2.2:1 (110:65/70 kd) and 4:1-10:1 (110:80 kd) (Vale et al., 1985c). (Note: k.l.p. molecular weight was typically determined using gel filtration chromatography, while subunit composition and molecular weights were typically determined using polyacrylamide gel electrophoresis). Therefore, the k.l.p.'s identified to date are composed of a major subunit of 110-135 kd, with some preparations also exhibiting the presence of smaller subunits. Reported molecular weights for the complete protein range from 400-600 kd. It is not surprising to see interspecies variation in the molecular weight of k.l.p.'s. Also, the different isolation methods mentioned here, although all based on microtubule affinity, could be responsible for the presence or absence of additional subunits.

Some recent work by Vale and Reese (1986) promises to shed light on this topic. Squid kinesin which was bound to microtubules with AMP-PNP and exposed to proteolytic enzymes (subtilisin, chymotrypsin, or elastase) yielded a 60 +/-3 kd fragment which remained bound to the microtubules through several wash steps. Further digestion, especially with subtilisin, generated fragments of 25 and 40 kd which also remained associated with the microtubules. While proteolytic fragments are unlikely to correspond directly to subunits, it is interesting that portions of the kinesin molecule retain their ability to bind to microtubules even when the majority of the molecule has been removed by digestion. The next logical step would be to bind kinesin to microtubules by different methods (e.g. ATP removal) and see if different portions of the molecule are left behind after digestion. This could provide hard evidence for the existence of two different types of microtubule binding, a "rigor" complex and a complex stabilized by the presence of a non-hydrolyzable analogue of ATP.

Vale et al., (1986b) have also recently shown that an 80 kd polypeptide is released from (squid?) kinesin bound to microtubules when ATP is introduced in the absence of Mg^{++}. This could correspond to the 80 kd subunit reported for squid optic lobe kinesin (the report was not clear on this point). Schroer and Sheetz (1986) have also used squid optic lobe kinesin in kinesin-affinity chromatography and have identified several proteins in squid optic lobe homogenate that bind to kinesin. Like kinesin, some of these proteins apparently show AMP-PNP dependent binding to microtubules. They postulate that kinesin could be part of an oligomeric axoplasmic motor complex. This could in fact explain the presence or absence of the smaller molecular weight subunits in the different types of k.l.p.'s; different parts of this postulated oligomeric complex may be present or absent in any given isolation. Unfortunately, they do not give the molecular weights of these kinesin-binding proteins, so no further conjecture may be made on this point. However, Schroer and Sheetz have also shown that kinesin will enhance the movement of partially purified squid axoplasmic vesicles on microtubules, but no movement is seen if highly purified vesicles are substituted in the preparation. It does, therefore, seem that more than simple kinesin is necessary for organelle movement, although simple kinesin is sufficient to allow these highly purified vesicles to bind to microtubules in the presence of AMP-PNP. Unfortunately, details of this last experiment were unavailable; no comment can be made on the identity or characteristics of "highly purified vesicles" (Schroer and Sheetz, 1986).

3. ATPase ACTIVITY:

One would expect an ATP-dependent mechanochemical enzyme to be an ATPase. However here, as in other aspects of kinesin's action, the story is not so simple. The original work done by Vale et al. (1985c) with squid and bovine kinesin stated that little ATPase activity (0.01 µmol/min/mg kinesin) eluted with the gel-filtration fractions that contained kinesin, although microtubule velocity as a function of [ATP] was later found to fit Michelis Menton kinetics (Schnapp et al., 1986). This low ATPase activity was activated 1-2 fold by 0.25% Triton X-100 or 0.8 M KCl; N-ethyl- maleimide (NEM) had no effect (Vale et al., 1985c). However Brady (1985) claimed he found ATPase activity preferentially associated with microtubules incubated in chick brain homogenate in the presence of AMP-PNP. This ATPase activity, present only in pellets containing chick brain k.l.p., displayed some of the properties previously shown to be related to axonal transport, such as lack of a Ca^{++} dependence and inhibition by EDTA in concentrations sufficient to chelate Mg^{++} in the buffer. The difference between the experimental designs of Vale et al. and Brady was the presence of microtubules in Brady's preparation. However, Scholey et al., (1985) noted no increase in ATPase activity which copelleted with microtubules incubated in sea urchin egg homogenate in the presence of AMP-PNP, even though a k.l.p. was identified.

This question has since been specifically examined by Kusnetsov and Gefland (1986), who isolated a k.l.p. from bovine brain using inorganic tripolyphosphate (PPP). This protein had a very low ATPase activity after elution from a gel filtration column - 0.06-0.08 µmol/min/mg in 3mM Mg^{++}. Though this activity was still very low, it was 6-8 times higher than that reported by Vale et al., (1985c) for squid kinesin. The difference could be due to the different methods used during purification, and may or may not be significant. When taxol-stabilized pure microtubules were added to Kusnetsov and Gefland's k.l.p.-containing eluant, the ATPase activity of the preparation increased dramatically to as high as 4.6 µmol/min/mg. The ATPase activity of pure taxol-stabilized microtubules was undetectable, so this increase in activity must have been due to an interaction between the k.l.p. and the microtubules. This microtubule-activated ATPase would hydrolyze other nucleotides at rates comparable to ATP hydrolysis (GTP - 81%; ITP - 61%; UTP - 41%; CTP - 85% c.f. rate of ATP hydrolysis). Strangely, kinesin eluted from their gel filtration column with no microtubules added had a substantial ATPase activity in the presence of 2 mM Ca^{++} (note: this is an unphysiologically high Ca^{++} level). More strangely, activation was higher in 2mM Ca^{++} than in 10 mM Ca^{++} (Vmax = 1.6 µmol/min/mg vs Vmax = 0.57 µmol/min/mg). Activation in 2mM

Ca^{++} required 80 times more ATP to reach 1/2 Vmax compared to activation by microtubules (Km = .0008 vs Km = .00001), again an unphysiological condition.

Penningroth et al., (1986) have also reported the activation of ATPase activity in the presence of microtubules and a k.l.p. isolated from bovine brain.

To further confuse the issue, Wagner et al., (1986) have recently reported the AMP-PNP- microtubule-affinity dependent isolation of a k.l.p. from bovine brain which shows "substantial ATPase activity" after purification on gel filtration and DEAE-Fractogel chromatography. The use of microtubules or Ca^{++} is not mentioned. Any final answers to the question of how kinesin acts as an ATPase must await further clarification. It is possible that species variation or differences in isolation techniques are responsible for the different results produced by different investigators. However, it is likely that kinesin or k.l.p.'s will act in the expected role of ATPase if the conditions are right; defining the conditions is trickier, but the presence of microtubules seems to be mandatory unless both Ca^{++} and a high concentration of ATP are present. The binding of kinesin to microtubules may induce a conformational change which exposes an ATPase site, or the microtubule itself may be part of the active site. The action of Ca^{++} is harder to explain. Since Kuznetov and Gefland are the only investigators to report this finding, and since they themselves do not comment upon it, this report will treat it as an anomaly, probably induced only by in-vitro conditions.

4. MOTILITY

One feature that should (and does, to some degree) unify the k.l.p.'s is their ability to induce movement - either of organelles or plastic beads on microtubules or of microtubules on glass. Motility would seem to be a crucial element for the identification of an alleged "kinesin", however not all investigators have been able to or have found it necessary to include this in their results. Piazzi and Stearns (1986) have shown that DU 145 human tumor cell k.l.p. will activate movement of carboxylated latex beads along microtubules; microinjection of this k.l.p. into living DU 145 cells induces directed saltatory movement of vesicles. Sea urchin k.l.p. which was frozen in liquid nitrogen and shipped to Vale and Sheetz in Woods Hole induced movement of microtubules on glass. This movement was later shown to be indistinguishable from that produced by neuronal kinesin (Scholey et al., 1985; Porter et al., 1986). A k.l.p. isolated from *Drosophila* causes microtubules to glide across glass in the same direction as induced by the squid and sea urchin proteins, corresponding to an anterograde direction (Saxton et al., 1986).

However, motility remains a bit of a mystery. In the initial purifications of kinesin carried out by Vale et al., (1985c), the following results were found: low speed supernatant (S1) from squid axoplasm would induce bidirectional organelle movement along microtubules, but high speed squid axoplasmic supernatant (S2) would induce movement in only one direction which was later shown to be the anterograde direction by Vale et al., (1985d). When this high speed supernatant had its microtubules removed by incubation with GTP and taxol (making S3), it would still cause unidirectional movement. Squid optic lobe homogenate and bovine brain homogenate, on the other hand, would not induce any kind of movement in either the S1, S2, or S3 state. Further purification of the squid optic lobe kinesin was performed by using AMP-PNP dependent microtubule affinity; this allowed the optic lobe kinesin to move microtubules on glass or move beads or organelles along microtubules in a unidirectional manner. An identical purification technique carried out on bovine brain homogenate, however, failed to release a motility causing factor. It was only after gel filtration chromatography that the bovine brain kinesin would finally cause movement. It is necessary to keep in mind here that these different "kinesins" all showed somewhat different subunit composition (see Figure 2, page 18); it is also possible that kinesin is less concentrated in squid optic lobe or bovine brain homogenate than in squid axoplasm. There may also be inhibitors present in brain which are not present in axoplasm; further purification may be necessary to remove these factors.

As stated earlier, squid axoplasmic S1 induces bidirectional movement of organelles along microtubules, whereas squid axoplasmic S2 and purified kinesin promote movement in only one direction. This raised two questions: in which direction does kinesin cause motility, and what causes transport in the opposite direction? To determine the polarity of kinesin action, an assay was developed using microtubules assembled on centrosomes in vitro (Vale et al., 1985d). Centrosomes are microtubule organizing centers found in many cells, including neurons. Centrosomes were purified and microtubules, with their plus ends free, were grown on them. It was found that kinesin bound to 0.1 µm latex spheres caused the spheres to move from the minus to the plus ends of microtubules, a direction corresponding to that of anterograde transport in intact axons.

Since S1 will promote bidirectional motility and the high speed supernatant S2 will not, Vale et al., (1985d) tried an alternative strategy. Vesicles and microtubules were removed from S1 by incubation in taxol (20 µM), GTP (1 mM) and Triton X-100 (0.5%) followed by a lower speed centrifugation (40,000 X g for 30 min). This S1a solution still promoted bidirectional movement of beads on microtubules. Much less movement of microtubules on glass was seen with S1a as compared to S2; proteins present in the S1a interfere with this kinesin generated movement. Retrograde bead movement promoted by S1a (1.4 µm/sec) was inhibited by N-ethylmaleimide and 20 µM vanadate while anterograde movement (0.6 µm/sec) was unaffected by these agents; S1a treated with these inhibitors would behave like S2, causing smooth unidirectional movements of beads on microtubules and microtubules on glass. This indicated that the retrograde translocator was pharmacologically distinct from kinesin. Also, the fraction containing retrograde movement collected at higher sucrose concentrations than kinesin during sucrose gradient centrifugation of S1. This showed either that the retrograde translocator was much larger than kinesin or that it tended to associate with large structures such as filaments. Organelles taken from the sucrose gradient showed bidirectional movement along microtubules at speeds similar to that of beads in the retrograde direction (1.5 µm/sec) but faster than beads in the anterograde direction (1.4 µm/sec). The difference in velocity may reflect a difference in the manner in which the anterograde and retrograde translocators interact with beads compared with organelles.

While interesting in itself, none of the above clarified the choice between the following two possibilities: kinesin or microtubules were either being modified in such a way that both pharmacological sensitivity and force direction were being changed, or there was another retrograde translocator involved. To distinguish between these possibilities, an affinity column was prepared using a monoclonal antibody against kinesin. S2 was applied to this column; the eluant from did not contain kinesin but did promote retrograde bead movement. These results indicated that there was a retrograde bead translocator which was pharmacologically and immunologically distinct from kinesin. This retrograde translocator was ATP-dependent, sensitive to trypsin, and inhibited by 20 µM vanadate, 2mM NEM or 7 mM AMP-PNP (Vale et al., 1985d).

An interesting twist has recently been added to this topic: Organelles travelling in myelinated axons ~~of~~ *Xenopus laevis* in both the anterograde or retrograde directions reversed direction when they reached the edge of a local 20 µM lesion in the axolemma. Normal retrograde transport was blocked by lower concentrations of vanadate (10 µM) and EDTA (5 mM) than reversed retrograde transport. Similarly, anterograde transport was unaffected by concentrations of these agents that inhibited reversed anterograde transport. This implies that individual vesicles switch driving mechanisms during reversal of rapid axonal transport (Smith, 1986). In fact, during rapid anterograde or retrograde transport in vertebrate axons, an organelle's velocity exhibits a slow periodic component of about 0.1 Hz, evidence that organelles are normally influenced by two oppositely-directed motive mechanisms (Smith, 1987). The slow variation in velocity could reflect a change in the proportional effect of two motors; perhaps turnaround at lesions is simply a dramatic example of the principle. Individual organelles moving on microtubules in crude axoplasmic extract rarely reverse their direction, unlike beads, which frequently reverse their direction (Vale et al., 1985d). It is likely that organelles contain receptors on their surface that recognize either the anterograde or retrograde translocator; beads lack these specific receptors. This hypothesis is strengthened by the fact that trypsin treatment of organelles abolishes their movement on purified microtubules even if active translocator is present (Schnapp et al., 1985). Direction reversal may require changes in the organelle's membrane; perhaps new surface proteins are exposed, or exposed proteins are modified. It would be interesting to use antibodies against kinesin to label organelles; if this were done it would show for the first time a direct relationship between kinesin and organelles, and would also provide a means of following the presence of this anterograde translocator during reversal.

5. PHARMACOLOGICAL STUDIES

The pharmacological properties of kinesin and k.l.p.'s are again rather controversial. Vanadate, an inhibitor of ciliary dynein, seems to be universially acknowledged as an inhibitor of kinesin-based motility. In extruded and dissociated squid axoplasm, 100 μM vanadate will block the movement of organelles or plastic beads along single microtubules, while 20 μM vanadate will not (Vale et al., 1985b). Microtubule movement promoted by purified squid or bovine kinesin is abolished by 100 μM vanadate and only slightly inhibited by 25 μM vanadate (Vale et al., 1985c). Vanadate at >50 μM will stop microtubule gliding promoted by k.l.p.'s isolated from sea urchin egg (Scholey et al., 1985) and *Drosophila* (Saxton et al., 1986). The microtubule-activated ATPase k.l.p. isolated from cow brain by Kuznetsov and Gefland (1986) is not inhibited by 2 μM vanadate, but ATPase action is inhibited 27% by 10 μM vanadate. Interestingly, in the S1a supernatant prepared by Vale et al., (1985d) which will support bidirectional movement of beads on microtubules, 20μM vanadate will inhibit retrograde bead movement (normally 1.4 μM/sec) but not anterograde bead movement (normally 0.6 μM/sec). This selective inhibition of retrograde transport by low concentrations of vanadate may also be seen in intact axoplasm (R.S. Smith, 1986, 1987).

N-ethylmaleimide (NEM), on the other hand, is subject to conflicting reports regarding its effect upon kinesin and k.l.p.'s. Preincubation with NEM causes modifications to -SH groups. Vale et al., (1985c) initially declared that while NEM inhibits dynein's ability to bind to microtubules and generate force, microtubule movement generated by kinesin was "largely unaffected" by 5 mM NEM. They later reported that retrograde movement of beads incubated in S1a was inhibited by 2mM NEM, with anterograde (kinesin-generated) motility remaining unaffected (Vale et al., 1985d). It seems odd, therefore, that Saxton et al., (1986) would later use the inhibition of microtubule gliding by [NEM] >10 mM as evidence that their k.l.p. from *Drosophila* was "fly kinesin". K.l.p. from sea urchin egg (Scholey et al., 1986) also displayed inhibition of microtubule gliding in [NEM] > 2 mM. The latest account from Porter et al., (1986) states that sea urchin k.l.p. and squid kinesin are both relatively insensitive to high concentrations of NEM; 3-5 mM are required to block microtubule motility on glass.

6. ANTIGENICITY

Another factor which attests to the similarity of kinesin and the k.l.p.'s is their shared antigenicities: antibodies to the sea urchin 134 kd subunit bind to squid 110 kd subunits (Scholey et al., 1985); antibodies to both 134 kd sea urchin and 110 kd squid subunits recognise the 115 kd *Drosophila* subunit, and vice-versa (Saxton et al., 1986); antibodies to the 134 kd sea urchin subunit will identify the 120 kd subunit from DU 145 human tumor cells (Piazza and Stearns, 1986). Vale et al. (1986a) have used antibodies to squid 110 kd subunits in a polyclonal immunoblot technique to identify k.l.p.'s in a number of mammalian tissue cultures (3T3 and PC12 cells), and have also used antibodies to squid 110 kd subunits to identify the presence of a k.l.p. in Chlamydomonas and Tetrahymena. It seems that the epitopes for kinesin are quite well preserved, which attests to the importance of the protein. Obviously, functions other than axonal transport are involved. In fact, antibodies to sea urchin kinesin have been used to stain the mitotic spindle in fixed sea urchin eggs (Scholey et al., 1985); when these antibodies are microinjected into dividing early sea urchin blastomeres, division is halted (Scholey et al., 1986). Antibodies against sea urchin k.l.p. that have been purified by immunoblots to DU 145 human tumor cell k.l.p. will stain the cytomatrix in non-dividing cells (Piazza and Stearns, 1986), which implies that kinesin and k.l.p.'s may be involved in general cytological functioning and not merely such specialized activities as axonal transport and mitosis.

Immunological techniques have also allowed researchers to begin probing the genetics of kinesin. Antibodies to 115 kd *Drosophila* k.l.p. have been used to screen a cDNA library prepared from fly heads; clones which encode for proteins recognized by this antiserum have been identified and isolated. Saxton et al., (1986) predict that these isolated clones encode for portions of *Drosophila* k.l.p..

7. ELECTRON MICROSCOPY

Electron microscopy has provided more clues to the structure and function of kinesin. The most extensive work in this field has been done by L.A. Amos (1987) who has worked with both shadowed and negatively stained specimens of a k.l.p. isolated from pig brain. According to her results, pig brain k.l.p., which is 100 nm long *in vitro*, consists of a rod 2-4 nm in diameter with a flexible joint in the middle. This rod has a large branched structure at one end consisting of 3-6 flexible stems with small heads at the end of each stem. At the other end, there is a small forked projection with two or possibly more prongs. When this k.l.p. is incubated with microtubules in the presence of AMP-PNP, the forked end attaches to microtubules, interacting with a small number of protofilaments, possibly only one; occasionally the large branched end is also seen bound to microtubules. She suggests that the subunit concentrations of this k.l.p. indicate that each projections of the large branched structure may correspond to one heavy (120 kd) chain. The small forked end may consist of two or more ATP-splitting heads, analogous to myosin, with the adjacent rod serving as a binding area for controlling proteins.

Electron microscopic observations of other investigators are consistent with these observations, although there are some differences. Shadowed squid kinesin was described as a "rod with a ball on one end and a smaller ball on the other" which was 50 - 70 nm long, a shorter length than porcine k.l.p., which could be explained by the lower molecular weight of the squid heavy chain (110 kd) (Vale et al., 1985c). However, Miller and Lasek (1985) and Gilbert et al., (1985) both reported that cross bridges between vesicles and microtubules had an average length of 17 nm and a maximum length of 30 nm, regardless of whether the vesicle was moving anterogradely or retrogradely at the time of fixation. As well as conflicting with the notion of different translocators for different directions, these measurements do not match well with kinesin's dimensions. It is possible that the cross bridges seen by Miller and Lasek (1985) and Gilbert et al., (1985) represent one half of the main 2-4 nm diameter rod; the rest of the rod may be attached to or aligned with the microtubule (Amos, 1987). It is also possible that these cross bridges represent other proteins, other motors that may be more permanently attached to vesicles (see Introduction, page 32).

8. MECHANICS OF KINESIN ACTIVITY

In a recent article, Hill (1986) used theoretical formalism from the study of muscle contraction to examine the mechanism of kinesin's action. A tentative kinetic diagram of kinesin's biochemical states was related to a plausible free energy diagram for these states. His proposals are summarized in Figure 3. It

Figure 3 : Proposed Kinesin Microtubule Interactions (Hill, 1986)

F = microtubules

A_1= kinesin, configuration 1

A_2= kinesin, configuration 2

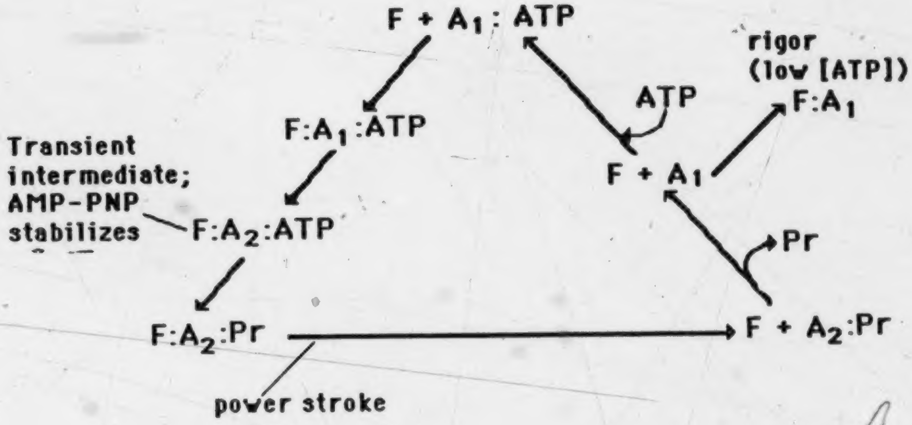
Pr= products: ADP, Pi

a) F:A_2:ATP is normally a transient intermediate, unless a non-hydrolyzable analogue of ATP is present. The conformational change from A_1 to A_2 allows rapid ATP hydrolysis; this change may occur in solution if ATP is present (hence the low-level solution ATPase activity of kinesin - Vale et al., 1985c). However, the presence of F catalyzes the A_1-A_2 conformational change and allows much higher rates of ATP hydrolysis. Separation of the F:A_2:Pr complex to F+A_2:Pr is necessary for the release of Pr.

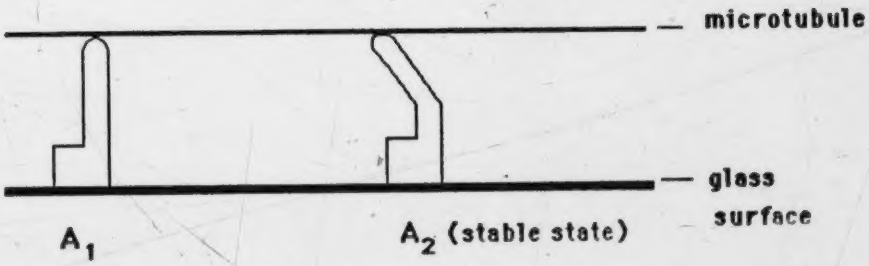
b) This is a schematic representation of microtubule movement on glass. After the transition F:A_1:ATP to F:A_2:Pr occurs, a force will be executed as A_2 shifts to its thermodynamically stable configuration.

Figure 3

a) ATPase cycle


F + A_1 : ATP
$F:A_1:ATP$
Transient intermediate; AMP-PNP stabilizes
$F:A_2:ATP$
$F:A_2:Pr$
power stroke
$F + A_2:Pr$
Pr
$F + A_1$
ATP
rigor (low [ATP])
$F:A_1$

b) Schematic Diagram


microtubule
glass surface
A_1
A_2 (stable state)

is interesting to compare this diagram to Figure 1 (Actin-Myosin and Microtubule-Dynein Interactions). Kinesin's ATPase cycle seems to run in the opposite direction to that of myosin's or dynein's.

Recent work by other investigators allows further comment upon the mechanism of kinesin's action. If there are two active sites, as Schnapp et al., (1986) hypothesize, several additions could be made to Figure 3. Dissociation of F:A2:AMP-PNP or F:A2:Pr to F+A2:Pr could be driven by ATP binding to the second active site. Since this dissociation can also be promoted by ADP + Mg^{++}, it seems actual hydrolysis at the second site is not necessary (Vale et al., 1986b).

The effect of Mg^{++}-depletion on this cycle is not clear. Depletion of Mg^{++} in the presence of ATP promotes the strong association of kinesin with microtubules (Vale et al., 1986b). Introduction of a 0 Mg^{++} buffer abruptly stops kinesin-induced microtubule movement on glass (Schnapp et al., 1986). The fact that there is no latency period before recovery of movement once Mg^{++} is reintroduced suggests that an F:A1-like rigor is involved (see Introduction, page 15).

9. OTHER POSSIBLE CYTOPLASMIC MOTORS

In addition to the true k.l.p.'s, other proteins have recently been discovered that share certain properties in common with the k.l.p.'s and should therefore be included in this discussion. Collins and Vallee (1986) have isolated a microtubule-activated ATPase from sea urchin eggs which associates with microtubules in the presence of ATP and is distinct from cytoplasmic dynein and kinesin. Basically, a high speed supernatant of egg homogenate was further separated by sucrose density centrifugation. A 10 S ATPase that was active only in the presence of microtubules was found. In contrast to dynein, no stimulation by nonionic detergents was exhibited. 100 μM sodium vanadate did not inhibit this ATPase (it will inhibit both dynein and kinesin activity) while NEM inhibits it completely (NEM inhibits dynein and has a controversial effect on k.l.p.'s). The fact that this protein differs from kinesin and dynein in sedimentation coefficient, microtubule binding characteristics, immunological specificity, and several pharmacological properties seems to indicate it represents a novel kind of ATPase. Whether it has a role in intracellular motility remains to be determined.

Lye et al., (1986) have identified a non-k.l.p. microtubule-based cytoplasmic motor in the nemotode *C. elegans* . This 20 S, 375 kd ATPase, which is isolated using a modified Vallee procedure, will transport microtubules across glass in an anterograde fashion at a speed of 0.8-1.0 μM/sec (c.f. 0.4 μM/sec for squid and bovine kinesin - Vale et al., 1985c). No binding agent is mentioned, although binding of this polypeptide to taxol-stabilized microtubules is ATP sensitive. Movement is ATP-dependent, and ATPase activity and microtubule motility are inhibited by [NEM]>1mM, [vanadate]>10μM, and activated by 0.2% Triton X-100.

Gilbert and Sloboda (1986) have isolated squid axoplasmic vesicles which translocate on isolated MAP-free microtubules in an ATP-dependent and trypsin-sensitive manner. When these vesicles were exposed to [å32-P]8-azidoadenosine 5'-triphosphate (a photoaffinity analogue of ATP), and UV light, a 292 kd polypeptide (which was later isolated in association with vesicles or taxol-stabilized microtubules) bound the analogue. This polypeptide cross-reacted with antiserum to porcine brain MAP-2. Because the 292 kd polypeptide binds ATP, which is associated with axoplasmic vesicles that translocate on MAP-free microtubules in an ATP-dependent fashion, can be isolated in association with vesicles and microtubules, and is released from microtubules in the presence of ATP, Gilbert and Sloboda believe this MAP-2 like polypeptide is involved in microtubule-vesicle interactions that promote vesicle motility.

Reticulomyxa is a giant freshwater ameba whose organelles exhibit rapid saltatory movements of up to 20 μM/sec when ATP is added to lysed preparations (Koonce and Schliwa, 1986). This lysed model system is also capable of other forms of motility, namely an active splaying of microtubule bundles and bulk streaming of organelle clusters. Reactivation does not occur with other nucleoside triphosphates, requires Mg^{++} ions, is insensitive to even high concentrations of EHNA {erythro-9-(3-[2-hydroxnonyl]) adenine}, is sensitive to vanadate only at concentrations greater than 0.1 mM, and is inhibited by N-ethylmaleimide at concentrations greater than 0.1 mM. The insensitivity to low concentrations of vanadate and EHNA is inconsistent with dynein-based motility. Nucleotide specificity (kinesin can use GTP - Vale et al., 1985c), velocity of organelle movement (an order of magnitude larger than kinesin) and sensitivity to low concentrations of NEM (>0.1mM vs. >3-5 mM for sea urchin or squid k.l.p.'s - see Porter et al., 1986) are inconsistent with k.l.p.-generated motility.

II. MATERIALS AND METHODS

A. MICROTUBULE PURIFICATION AND CHARACTERIZATION

1. INTRODUCTION

In order to perform microtubule-affinity purification of bovine k.l.p., it was necessary to obtain pure microtubules. To obtain the best purification method, the literature was searched for information on these polymeric filamentous proteins. The existence of cytoplasmic microtubules was recognized in 1963, when glutaraldehyde fixatives were first used in electron microscopy (Ledbetter and Porter, 1963). Large numbers of microtubules are present in neural tissue (Wuerker and Kirkpatrick, 1972). Microtubules are tubular structure 20-26 nm in diameter and are formed by the polymerization of the protein tubulin, which is composed of alpha and beta subunits. Typically the wall of the tubule is approximately 6 nm thick; the center is generally clear (Peters et al., 1976). The walls are thought to be composed of 13 globular subunits (Tilney et al., 1973). Microtubules are in equilibrium with a pool of unassembled monomers, and use the energy of GTP hydrolysis during assembly. This energy input allows very rapid polymerization. The polymerized tubulin maintains an intrinsic polarity - one end of the polymer contains exposed alpha subunits, while the other end contains exposed beta subunits. The filaments assemble spontaneously to give long uniform polymers held together by noncovalent interactions. Also, at the 'A' or assembly end, subunits are added more rapidly than they are lost, wheras at the 'D' or disassembly end, the converse is true. Therefore, the microtubule is not a static entity, but is being constantly "treadmilled" (*Methods in Cell Biology*, Vol. 24).

2. PURIFICATION TECHNIQUE

a. Taxol

Taxol is an antimitotic agent derived from the western yew plant. It is effective in promoting microtubule assembly at a close to 1:1 molar stoichiometry to tubulin, the principle subunit of the microtubule polymer (Schiff & Horwitz; 1980). Taxol mimics, to some extent, the effect of the microtubule-associated proteins (MAPs), which promote the assembly of tubulin in vitro, and possibly in vivo (Olmsted & Lyon, 1981).

We used taxol to purify bovine microtubules according to the procedure developed by Vallee (1982). He found that extensive microtubule assembly occured in the presence of taxol at 37°C, 0°C, and at 37°C with 0.35 M NaCl. Assembly is normally inhibited under the latter two conditions if taxol is not present. At 37°C and 0°C, complete assembly of both tubulin and the MAPs was observed in the presence of taxol. However, at elevated ionic strength, only tubulin assembled, forming microtubules devoid of MAPs. The MAPs could also be released from the surface of preformed microtubules by exposure to elevated ionic strength. The yield of tubulin from white matter was 57% of that from grey on the basis of tissue wet weight.

b. Procedure (see Figure 4)

Cow brain was obtained from a slaughterhouse (Gainers Inc., 12425-66 St., Edmonton, Alberta). Brains were removed within 10 minutes of the animals' deaths. Intitially the heads were bisected medially along the sagittal plane with a band saw. This make the entire head commercially useless, so later specimens were first cut along frontal and transverse planes to remove the portion of the head containing the brain; this portion was then bisected medially

Figure 4 : Purification of Microtubules

Bovine brain was removed less than 10 min after the animals' deaths and placed in 4°C PEM (100 mM Pipes - pH=6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$). Grey matter was isolated and homogenized in 1.5 volume of PEM buffer.

1) Centrifugation of homogenate (5°C) yields supernatant S_1 and pellet P_1. P_1 is discarded.
2) High speed centrifugation of S_1 (5°C) yields S_2 and P_2. P_2 is discarded.
3) Incubation of S_2 with 1.0 mM GTP and 20 µM taxol for 20 min at 23°C polymerizes microtubules. Centrifugation yields S_3 and P_3. S_3 is discarded.
4) P_3, containing endogenous microtubules, is resuspended in 1/10 original S_1 volume (O. S1 V.) of PEM + 20 µM taxol + 1 mM GTP + 1.0 M NaCl and incubated for 15 min at 23°C. This step removes microtubules associated proteins (MAPs) from the microtubules. The solution is layered on top of a 5% sucrose cushion and centrifuged, yielding S_4 and P_4. S_4 is discarded.
5) P_4, containing MAP-less microtubules, is resuspended in 1/20 O. S_1 V. of PEM + 20 µM taxol + 1 mM GTP and incubated for 15 min at 23°C. This final wash serves to desalt the microtubules. The solution is centrifuged, yielding S_5 and P_5. S_5 is discarded.

P_5, containing clear microtubules, is resuspended in enough PEM + 20 µM taxol + 1 mM GTP to give a 25 mg tubulin/ml final concentration.

Centrifugation, unless otherwise stated, was at 37,000 X g for 30 min at 23°C using a Servall SS-34 rotor in a Servall RC-2 centrifuge. High speed centrifugation (step 2) was at 100,000 X g for 120 min using a Beckman type 40 rotor in a Beckman L3-50 ultracentrifuge.

along the sagittal plane. The brains were immediately placed in 4°C PEM buffer [100 mM piperazine - N,N-bis (2 ethane sulfonic acid), pH 6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$] and transported to our lab. White and grey matter were manually separated on an ice-cold surface using a #22 blade scalpel. The grey matter was homogenized for 5 bursts of 5 sec in 1.5 volume of PEM buffer, using a Virtis 45 homogenizer. The homogenate was centrifuged at low and then high speed to remove cell debris. Microtubules were polymerized by the addition of 1 mM GTP and 20 μM taxol. The newly-formed filaments were centrifuged out of solution by low speed centrifugation, washed in a high-salt buffer to remove MAPs, desalted, and resuspended in PEM + 20 μM taxol + 1 mM GTP. Aliquots were frozen in liquid N_2 and stored at -70°C. We isolated a total of 74.8 mg of clean microtubules from 72 g of grey matter (0.1 % yield).

3. CHARACTERIZATION

a. Electron Microscopy - Thin Section Transmission Electron Microscopy (TEM)

Microtubules (25 mg/ml) purified from bovine brain were centrifuged in a Beckman Microfuge B (cat.#338720). The pellet was fixed overnight with 3% glutaraldehyde in a 0.05 M cacodylate buffer (pH = 7.0) and then treated according to the following schedule:

post-fixation	1) 0.1 M cacodylate buffer (pH 7.0)	3 X 10 min
	2) 1.0% osmium tetroxide in phosphate buffer (pH 7.0)	1 X 30 min
	3) ddH$_2$O (double-distilled water)	3 X 5 min
graded dehydration	4) 50% Etoh	1 X 15 min
	5) 75% Etoh	1 X 15 min
	6) 95% Etoh	1 X 15 min
	7) 98% Etoh	1 X 15 min
	8) Absolute Etoh	1 X 10 min
embedding	9) Propylene oxide	2 X 10 min
	10) Propylene oxide Poly/Bed 812 (Polysciences) (1:1 by volume)	overnight
	11) Poly/Bed 812 (put with sample into mold)	2 days (in 60°C oven)

The blocks were then thin sectioned. Sections were collected on a 300-mesh grid, stained with uranyl acetate and lead citrate, and examined with a Siemens 102 EM.

b. Electron Microscopy - Negative Staining

A small amount of 3% glutaraldehyde in 0.05 M cacodylate buffer (pH = 7.0) was added to a 25 mg/ml solution of bovine brain microtubules (approx. dilution of sample = 1:3). A drop of this solution was pipetted onto a Formvar - coated 200 mesh grid and left for about 10 sec. The grid was then held at an angle and a few drops of 2% aqueous sodium silicotungstate were run across the grids' surface. Extra fluid was removed by touching the edge of the grid with filter paper. The grid was then examined on a Siemens 102 EM.

c. Scanning Electron Microscopy

A plastic coverslip was incubated for a few minutes in a 0.5% gelatin solution. It was then removed and allowed to dry. One drop of clean microtubule solution (25 mg/ml) (see Figure 4, page 39) was placed on the gelatin-coated coverslip, allowed to adhere for 30 sec, then covered with a drop of primary fixative (2.0% gluteraldehyde) for 15 min at room temperature. Excess fluids were removed with a piece of tissue applied to the edge of the coverslip. The fixed microtubules were then rinsed with 0.1 M cacodylate and incubated for 15 min at room temperature in 1% osmium tetroxide in 0.1 M cacodylate. The sample was then rinsed several times in 0.1 M caodylate, followed by several rinses in distilled water. The sample was then incubated in a fresh saturated solution of thiocarbohydrazide in water for 15 min, followed by a 15 min incubation in the 1% osmium tetroxide - 0.1 M cacodylate solution and several 0.1 M cacodylate and distilled water rinses. Then the thiocarbohydrazide, 1% osmium tetroxide - 0.1 M cacodylate, and 0.1 cacodylate/distilled water steps were repeated one time. The sample was then dehydrated in a graded series of acetone 2 min incubations (25, 50, 70, 90, and 100%). Critical point drying was performed using liquid CO_2. Scanning electron microscopy was performed with a Phillips SEM 505 Scanning Electron Microscope.

d. Video-Enhanced Differential Interference Contrast Microscopy

A #1-22 X 22 mm coverslip was given a thin border of grease. Two µl of squid kinesin solution (about 66 µg/ml - kindly provided by Bruce Schnapp, Marine Biological Laboratory, Woods Hole, MA) was placed in the center of the coverslip. This kinesin will bind to glass coverslips and normally will promote the motility of microtubules along the glass surface (Vale et al., 1985c). Two µl of microtubule solution (25 mg/ml) was added, along with 2 µl of 10 mM ATP solution. The preparation was placed on a slide and viewed using video-enhanced differential interference contrast microscopy (provided by Dr. Smith). We had been warned by Bruce Crise, a technician in Bruce Schnapp's lab, that this kinesin solution was quite old and would probably not promote motility. No motility was seen, but the preparation technique caused microtubules to stick to the coverslip, which made the microtubules highly visible.

B. K.L.P. PURIFICATION AND CHARACTERIZATION

1. INTRODUCTION

Kinesin and kinesin-like proteins have been isolated from a variety of tissues (see Figure 2, page 18). Except for the use of monoclonal antibody affinity columns (Vale et al., 1985d), isolation techniques have been based upon the principle of microtubule affinity purification (first outlined by Vale et al., 1985c).

Our initial isolations were performed using white matter from cow brain, following the procedure of Vale et al., (1985c). Later, modifications were made to the procedure in order to simplify it, to use different tissue sources, to increase the the yield of k.l.p., to increase the purity of the isolate, or to try new methods of binding k.l.p. to microtubules.

2. ISOLATION TECHNIQUE

a. Microtubule Affinity Purification of K.L.P. (from Vale et al., 1985c) (see Figure 5)

Cow brain was obtained from a slaughterhouse (Gainer's Inc., 12425-66 St., Edmonton, Alberta). Brains were removed within 10 minutes of the animals' deaths. Initially the heads were bisected medially along the sagittal plane with a band saw. This made the entire head commercially useless, so later specimens were first cut along frontal and transverse planes to remove the portion of the head containing the brain; this portion was then bisected medially along the sagittal plane. The brains were immediately placed in 4°C saline solution (9% NaCl) and brought to the lab.

Figure 5 : Microtubule-Affinity Purification of K.L.P. : Procedure of Vale et al., (1985c) *(Variations to the procedure of Vale et al. are printed in italics)*

Bovine brain* was removed less than 10 min after the animals' deaths and placed in 4°C saline. White matter was isolated and homogenized in 1.5 volume of PEM buffer(100 mM Pipes - pH=6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$).

1) Centrifugation of homogenate (5°C) yields supernatant S_1 and pellet P_1. P_1 is discarded.
2) High speed centrifugation of S_1 (5°C) yields S_2 and P_2. P_2 is discarded.
3) Incubation of S_2 with 1.0 mM GTP and 20 µM taxol for 20 min at 23°C polymerizes microtubules. Centrifugation yields S_3 and P_3. P_3 is discarded.
4) S_3 is incubated for 15 min at 23°C with clean microtubules (100 µg/ml) and a binding agent (B.A.)**. Centrifugation yields S_4 and P_4. S_4 is discarded .
5) P_4, containing microtubules and k.l.p., is resuspended in 1/10 Original S_1 Volume (O. S_1 V.) of PEM (100 mM Pipes - pH=6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$) + B.A. + 20 µM taxol, and incubated for 15 min at 23°C. Centrifugation yields S_5 and P_5. S_5 is discarded. ***
6) P_5, containing microtubules and k.l.p., is resuspended in 1/20 O.S_1V.**** of PEM + 5 mM ATP + 0.1 M KCl + 20 µM taxol and incubated for 40 min at 23°C. Centrifugation yields S_6 and P_6. P_6 is discarded or re-extracted with PEM + 5 mM ATP + 0.1 M KCl. S_6 contains k.l.p..

No B.A. was added to control solutions. *As an additional control, microtubules were occasionally not added at Step 4.*

Figure 5 continued

* *Other tissues homogenized included rabbit kidney and liver, cow kidney and liver, pig brain and salivary gland, and dog brain and spinal cord.*

**Vale et al., (1985c) used 5 mM AMP-PNP as a B.A.; *we have also used 5-10 mM PPP (Kuznetsov and Gefland, 1986) and 5-100 mM EDTA as B.A..*

****We tried eliminating this wash step (see Results - Run #2) and also tried adding another wash step (see Results - Run #4). When an additional wash step was tried,* P_5 *was resuspended in 1/20 O.* S_1 *V. PEM + 20 μM taxol, incubated for 15 min at 23°C, and centrifuged, yielding* P_5a and S_5a. S_5a was *discarded and* P_5a was *processed as* P_5 *normally would be (step 6).*

*****Different* S_1:S_6 *ratios were tried, ranging from 1:20 to 1:60.*

Centrifugation, unless otherwise stated, was at 37,000 X g for 30 min at 23°C using a Servall SS-34 rotor in a Servall RC-2 centrifuge. High speed centrifugation (step 2) was at 100,000 X g for 120 min using a Beckman type 40 rotor in a Beckman L3-50 ultracentrifuge.

White matter was manually separated from the rest of the brain on an ice-cold surface using a #22 blade scalpel. Two different methods of homogenizing the white matter were attempted. Initially, a Virtis-45 homogenizer was used for 5 bursts of 5 sec. Some problems were experienced in obtaining an even homogenate, so we switched to 5 bursts of 5 sec on a Polytron type PT10/35; this gave better results.

White matter was homogenized at 4°C in a weight:volume ratio of 1:1 with homogenization buffer. Initially, we used the same homogenization buffer used by Vale et al., (1985c). As more groups performed isolations on a variety of tissues, it became apparent that a variety of buffer recipes were possible (see following section).

After homogenization, the homogenate was centrifuged at low and then high speed to remove cell debris. Microtubules were polymerized by the addition of 1 mM GTP and 20 µM taxol and centrifuged out of solution. Then clean microtubules and a k.l.p. binding agent (B.A.) were added. Vale et al., (1985c) used 5 mM AMP-PNP as a binding agent; we have experimented with others (see next section). The microtubules with bound k.l.p. were centrifuged out of solution and washed in a B.A. containing buffer. The k.l.p. was then released by resuspending and incubating the pellet in a buffer containing 5 mM ATP + 0.1 M KCl + 20 µM taxol. The microtubules were removed by centrifugation and the supernatant containing released bovine translocator was collected. Several variations to this procedure were also attempted (see Figure 5, page 45; also see next section).

b. Variations on the Isolation Procedure of Vale et al., (1985c)

(i) Homogenization Buffer

As mentioned above, as more groups performed k.l.p. isolations, it became apparent that a variety of homogenation buffer recipes were possible. Following is a list of the different homogenization buffers used by researchers in the field:

Vale et al., (1985c) Cow Brain

50 mM Pipes pH = 7.0
50 mM Hepes
2 mM $MgCl_2$
1 mM EDTA
0.5 mM DTT
1 mM PMSF
10 µg/ml leupeptin
10 µg/ml TAME
0.5 mM ATP

Kuznetsov and Gefland, 1986 Cow Brain

50 mM imidazole HCl pH = 6.7
0.5 mM $MgCl_2$
0.1 mM EDTA
1.0 mM 2 - mercaptoethanol

1 mM PMSF added after first centrifugation

PMSF added to all subsequent buffers - 0.1 mM

Amos, 1987 Pig Brain

0.1 M Pipes pH = 6.9
2.0 mM $MgCl_2$
1.0 mM EGTA
0.5 mM DTT
1.0 mM GTP
0.5 mM ATP
0.1 mM PSMF
0.2 µg/ml pepstatin A
1.0 µg/ml TAME
2.0 µg/ml leupeptin

Scholey et al., 1985 Sea Urchin Eggs

0.1 M piperazine pH = 6.9
2.5 mM $Mg(CH_3COO)_2$
5.0 mM EGTA
0.1 mM EDTA
0.9 M glycerol
0.1 M PMSF
1.0 µg/ml pepstatin
1.0 µg/ml leupeptin
10 µg/ml aprotinin
0.5 mM DTT

Brady, 1985 Chick Brains

0.1 M Pipes pH = 6.94
1.0 mM EGTA
1.0 mM $MgCl_2$
1.0 mM GTP
0.5 mM ATP

Based upon the above homogenization buffers, we developed our own buffer which contained:

0.1 M Pipes pH = 7.0
5.0 mM EGTA
1.0 mM $MgCl_2$
1.0 mM DTT
5.0 µg/ml leupeptin
5.0 µg/ml TAME
0.1 mg/ml soybean trypsin inhibitor

This buffer was similar to the original used by Vale et al., (1985c) with the following changes: EDTA was eliminated since Mg^{++} was present in the buffer and EDTA chelates Mg^{++}; PMSF was eliminated, since it would not dissolve; ATP was eliminated since its presence could have increased the amount of AMP-PNP necessary during k.l.p. purification; and soybean trypsin inhibitor was added, as a replacement for PMSF.

It should be noted that using simple PEM buffer (0.1 M Pipes, 5.0 mM EGTA, 1.0 mM $MgSO_4$, pH = 6.6) for the homogenization worked well (see Results-Run #1). However, homogenate prepared with PEM buffer, frozen in liquid N_2 and stored at -70°C for 2-3 weeks showed poorer results when k.l.p. isolations were performed. Homogenate prepared with homogenization buffer and treated in the same way remained relatively stable. This is probably because of the protease inhibitors present in homogenization buffer.

(ii) Other Variables (see Figure 5, page 45, and Figure 6, page 53)

Tissues other than bovine brain were used for k.l.p. purifications, including rabbit kidney and liver, cow kidney and liver, pig brain and mandibular gland, and dog brain and spinal cord. Results and conclusions for all but dog brain will be discussed only in general terms (see Discussion, page 147).

Dog brain was obtained from the Surgical Medical Research Institute (S.M.R.I.) at the University of Alberta. Brains were removed from freshly killed dogs by skinning the head and removing the dorsal part of the skull with a hammer and chisel.

We also introduced a number of variables into the isolation procedure itself. EDTA chelates Mg^{++}. The correct tertiary orientation of the ATP molecule during ATPase binding depends upon this divalent cation. Therefore, it seemed logical to try using EDTA as a means of binding k.l.p. to microtubules. The prevention of the normal configuration of ATP could possibly create either a rigor-like condition or a non-hydrolyzable situation (see Introduction, page 12). We therefore used EDTA in place of AMP-PNP, keeping all other steps unchanged. Since $MgSO_4$ was present in the homogenization buffer and since EDTA-promoted kinesin binding was based upon the Mg^{++}-chelating properties of EDTA, we expected that large quantities of EDTA would be necessary. Isolations were performed with a variety of EDTA concentrations in order to determine the levels of EDTA necessary for effective k.l.p. purification. Before these results were published, the fact of strong kinesin-microtubule binding induced by Mg^{++} depletion was reported by other investigators (Vale et al., 1986b). In other experiments, sodium tri-polyphosphate (PPP) was also used as a substitute for AMP-PNP (Kuznetzov and Gefland, 1986).

We thought that there could be a possible loss of k.l.p. in the wash step; therefore, we tried eliminating this step. No noticable increase in k.l.p. was seen, although the presence of contaminating proteins was greatly increased (see Results-Run #2). This variation was therefore abandoned. In an attempt to further reduce the concentration of contaminating proteins, an additional wash was also tried. This had little effect on the concentration or purity of k.l.p. (see Results-Run #4).

Another attempt to increase the final concentration of k.l.p. was altering the ratio between S_1 and S_6. This would theoretically release the k.l.p. collected from a fixed amount of crude homogenate into a smaller volume of final buffer. Normally a 20:1 ratio was used, but ratios up to 60:1 were tried. This did not seem to substantially increase k.l.p. concentration (see Results: 40:1-Runs #5,6,7; 60:1-Run #8).

Another variation was the use of endogenous microtubules in place of separately purified and cleaned microtubules. White matter was homogenized, centrifuged at 25,000 X g for 30 min at 4°C and recentrifuged at 150,000 X g for 60 min at 4°C. GTP (1 mM) and taxol (10 µM) were added to the supernatant, followed by a 30 min incubation at 25°C. At this point, the binding agent (AMP-PNP, EDTA, or PPP) was added and the homogenate was incubated for 30 min. In this way, the freshly polymerized microtubules were used for kinesin attachment rather than simply being removed and discarded. The microtubules were pelleted at 37,000 X g for 20 min at 20°C, the supernatant was discarded, and the pellet was processed as per normal (see Figure 6).

In an attempt to visualize bovine k.l.p. on microtubules, P_5 (see Figure 5, page 45) from a purification run (B.A. = 5 mM AMP-PNP, 1 wash, clean microtubules, S_1:S_6 = 20:1) was not resuspended in an ATP-containing buffer, but was treated as detailed elsewhere (see Materials and Methods, page 41) and viewed by TEM. No bovine k.l.p. could be seen attached to these microtubules, possibly because of the low concentration of bovine k.l.p. in our S_6 (see Discussion, page 142). Further pursuit of this avenue of investigation would likely be profitable.

Figure 6 : Microtubule-Affinity Purification of K.L.P. : Using Endogenous Microtubules *(variations from Figure 5 are printed in italics)*

Bovine brain was removed less than 10 min after the animals' deaths and placed in 4°C saline. White matter was isolated and homogenized in 1.5 volume of PEM buffer(100 mM Pipes - pH=6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$).

1) Centrifugation of homogenate (5°C) yields supernatant S_1 and pellet P_1. P_1 is discarded.
2) High speed centrifugation of S_1 (5°C) yields S_2 and P_2. P_2 is discarded.
3) Incubation of S_2 with 1.0 mM GTP and 20 μM taxol for 20 min at 23°C polymerizes microtubules. *Note: no centrifugation is performed, so no* S_3 *or* P_3 *are produced.*
4) The S_2 containing polymerized microtubules is incubated for 15 min at 23°C with binding agent (B.A.)*. Centrifugation yields S_4 and P_4. S_4 is discarded.
5) P_4, containing microtubules and k.l.p., is resuspended in 1/10 Original S_1 Volume (O. S_1 V.) of PEM (100 mM Pipes - pH=6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$) + B.A. + 20 μM taxol, and incubated for 15 min at 23°C. Centrifugation yields S_5 and P_5. S_5 is discarded.
6) P_5, containing microtubules and k.l.p., is resuspended in 1/20 O. S_1 V.** of PEM + 5 mM ATP + 0.1 M KCl + 20 μM taxol and incubated for 40 min at 23°C. Centrifugation yields S_6 and P_6. P_6 is discarded or re-extracted with PEM + 5 mM ATP + 0.1 M KCl. S_6 contains k.l.p..

No B.A. is added to control solutions.

Figure 6 continued

* B.A. = 5 mM AMP-PNP, 40 mM EDTA or 5-10 mM PPP

** Different S_1:S_6 ratios were tried, ranging from 1:20 to 1:60.

Centrifugation, unless otherwise stated, was at 37,000 X g for 30 min at 23°C using a Servall SS-34 rotor in a Servall RC-2 centrifuge. High speed centrifugation (step 2) was at 100,000 X g for 120 min using a Beckman type 40 rotor in a Beckman L3-50 ultracentrifuge.

3. FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

a. Introduction

In molecular exclusion chromatography (also known as gel-filtration or molecular-sieve chromatography) the mixture of proteins, dissolved in a suitable buffer, is allowed to flow by gravity down a column packed with beads of an inert, highly hydrated polymeric material that has previously been washed and equilibrated with the buffer alone. Common column materials include Sephadex, the commercial name of a polysaccharide derivative; Bio-Gel-P, a commercial polyacrylamide derivative; and Bio-Gel-A, composed of agarose (another polysaccharide) - all of which can be prepared with varying degrees of internal porosity. In the column, proteins of different molecular size penetrate into the internal pores of the beads to differing degrees. Very large molecules which never enter the bead's pores move through the chromatographic bed fastest. Smaller molecules, which can enter the bead's pores, move more slowly through the column. Molecules are therefore eluted in order of decreasing molecular size. Proteins of known molecular weight may be run to standardize the column; the molecular weight of unknown proteins may then be characterized.

Normally, with gel filtration, resolution decreases with increase in flow rate. If gravity feed is used, maximum resolution requires a long column and a low flow rate. The optimal flow rate for gravity-fed Sephadex columns, for example, is about 2 $ml/cm^2/hr$. In contrast, optimum separation with Superose columns using High-Performance Liquid Chromatography (HPLC) systems is obtained at 18-30 $ml/cm^2/hr$. This rapidity is allowed by the use of high pressure within the column which increases the Brownian motion of solute molecules, thereby increasing the degree of interaction between the solute molecules and the porous beads. Superose is a cross-linked, agarose-based medium with a narrow particle size distribution which enables high flow rates at low back pressures. Columns with Superose can be used with any HPLC system at pressures up to 1.5 M Pa (15 bar, 215 psi) for Superose 6 and 3 MPa (30 bar, 430 psi) for Superose 12. Different particle sizes (Superose 6: 13 µm; Superose 12: 10 µm) give these two gels different optimal separation ranges (for globular proteins - Superose 6: 5,000 - 5,000,000; Superose 12: 1,000 - 300,000).

Since squid kinesin had a reported molecular weight of 600,000 (Vale et al., 1985c), we chose to work with a Superose 6 column. This column was used with the Pharmacia FPLC system, which is specially designed for fast, high resolution chromatography of proteins, polypeptides, polynucleotides, and other biopolymers. Solvent delivery is powered by two high precision P-500 Pumps controlled by the Gradient Programmer GP-250. Complex gradient methods using multiple solvents may be programmed into the GP-250. The absence of stainless steel in contact with solutions insures samples remain uncontaminated by heavy metal ions. Samples are injected manually via the Valve V-7. Detection (UV monitor UV-1) is recorded (Chart Recorder Rec 482) and eluant is collected in either a time/volume mode or a peak-cutting mode by a programmable fraction collector (Frac-100).

b. Procedure

(i) Column Standardization

Proteins of known molecular weight were run on the column in order to correlate elution volume with solute molecular weight. Two hundred µl of 1 mg/ml solutions of Thyroglobulin (669 kd; elution volume=12.2 ml), Apoferritin (480 kd;15.0 ml), Human 8 globulin (160 kd;16.5 ml), Bovine Serum Albumin

(67 kd;17.3 ml), and Ovalbumin (45 kd;18.0 ml) were injected onto a Pharmacia Superose 6 column (gel buffer: 0.1 M KCl, 0.05 M Tris, 5 mM $MgCl_2$, pH = 7.6). A Pharmacia FPLC system was used for buffer delivery at 0.4 ml/min. Proteins were detected by a Pharmacia Single path UV-1 monitor (280 nm, 0.05 optical density) (see Figure 7). The detection system was also calibrated with Bovine Serum Albumin. Two times 100 µl of 5, 10, 20, and 40 µg/ml Bovine Serum Albumin were injected onto a Pharmacia Superose 6 column (gel buffer: 0.1 M KCl, 0.05 M Tris, 5 mM $MgCl_2$, pH = 7.6). A Pharmacia FPLC system was used for buffer delivery at 0.4 ml/min. Proteins were detected by a Pharmacia Single path UV-1 monitor (280 nm, 0.05 optical density) (see Figure 8).

(ii) Buffers

Initially we used a standard phosphate column buffer (*Data for Biochemical Research*, 2nd Edition, Dawson et al., 1969). However, high concentrations of phosphate inhibit kinesin-generated organelle movement (Vale et al., 1985c) and since we wanted to try motility tests, we changed our buffer to that used by Piazza and Stearns (1986) and Vale et al. (1985c) (0.1 M KCl, 0.05 M Tris, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA pH = 7.6). It seemed odd for $MgCl_2$ and EDTA to be in the same buffer, since EDTA chelates Mg^{++}. The function of ATP was equally questionable. We therefore initially tried running S_6 on the column using a buffer (0.1 M KCl, 0.05 M Tris, 5 mM $MgCl_2$ pH = 7.6) without ATP or EDTA. Using this buffer, we seemed to be losing about 80-90% of our bovine k.l.p. during column transit. The addition of ATP and EDTA did not alleviate this condition; the only difference was a much higher baseline readout from the spectrophotometer (due to the high molar absorption coefficient of ATP).

Figure 7: Superose 6 - Elution Volume vs log Molecular Weight

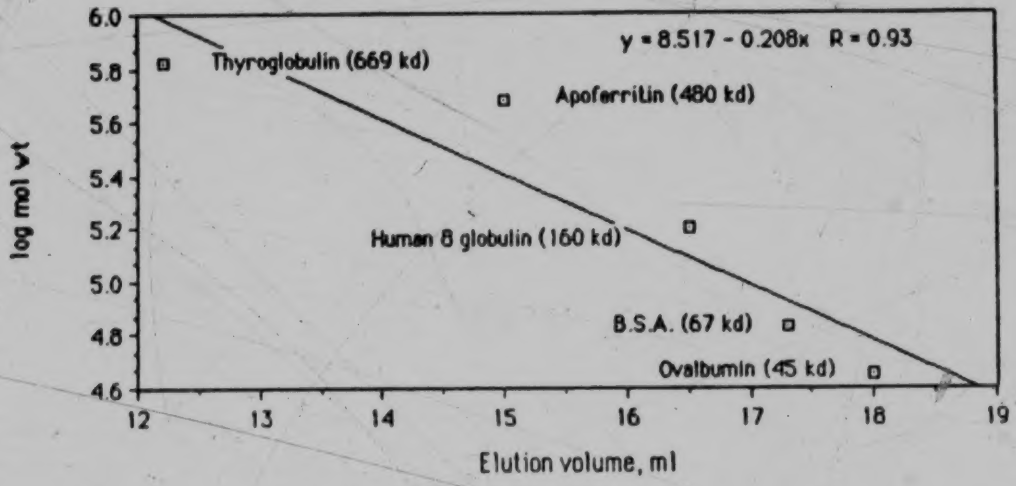
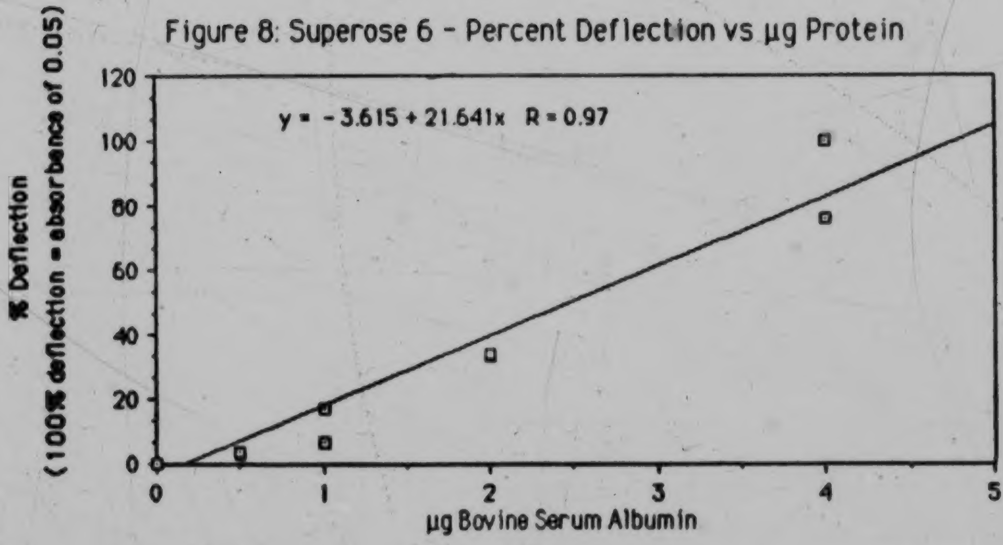


Figure 8: Superose 6 - Percent Deflection vs µg Protein



In another attempt to release the k.l.p. apparently being lost on the column, SDS (sodium dodecyl sulfate) was used in the column buffer. Kinesin sticks to glass coverslips (Vale et al., 1985c) and since the walls of the Superose 6 column were also glass, a way was sought to prevent k.l.p. binding. We had reason to suspect that bovine k.l.p. was positively charged (see Discussion, page 134) and hoped that the negatively charged groups of SDS would swamp the bovine k.l.p. and prevent it from binding to the glass walls of the Superose 6 column. Originally, we tried adding 0.1% SDS to the Tris buffer but this caused a precipitate to form. The critical miscelle concentration (CMC) of SDS is 8.27 mM, and 0.1% SDS is only 3.47 mM. Apparently the 0.1 M KCl in the Tris buffer increased hydrophobic interactions between the free detergent molecules, and lowered the CMC of the SDS. Therefore, an ammonium acetate buffer (0.1 M acetate brought to pH = 6.4 with ammonium hydroxide) was used allowing the 0.1% SDS to remain in solution.

The SDS did not seem to make a difference in the release of kinesin, and it caused column back pressure to rise continually throughout the run. Column washing also became necessary after each run. Since bovine k.l.p. losses did not seem to be solved by this approach, we abandoned it.

Our attempts to stop column losses of bovine k.l.p. were unsuccessful. Despite this, bovine k.l.p. was recovered in sufficient quantities from column eluant to allow its immunological identification and M.W. characterization. However, column eluant k.l.p. concentration was too low to promote the motility of latex beads on microtubules (see Materials and Methods, page 76).

(iii) After-Column Concentration

Another problem we encountered was the concentration of bovine k.l.p. after it had been eluted from the column. If the protein being collected was eluted in a concentrated peak, it could be collected into approximately a 2 ml sample; if a 200 µl sample had originally been injected onto the column, the dilution factor was 10:1 (column eluant : original sample). Three methods were tried for concentrating the k.l.p. in the column eluant: centricon microconcentrators, freeze-drying and adsorption to glass wool.

a) Centricon

Principle

Concentration of macromolecules in solution is achieved by ultrafiltration through a low-absorption, hydrophilic YM membrane, which is available with either 10,000 (Centricon10) or 30,000 (Centricon 30) molecular weight cutoff. The filtrate is collected in a filtrate cup. Macromolecular solutes are retained and become concentrated in the sample reservoir. These concentrators are capable of an 80-fold enrichment of the sample.

The driving force for filtration is provided by centrifugation at 1,000-5,000 X g, achieving transmembrane pressures of up to 111 psi. The microconcentrator was used in a fixed-angle rotor (Servall Angle Centrifuge-1017) which, during centrifugation, provides a centrifugal force vector at a constant angle to the membrane surface. As sample concentration progresses, this angle causes retained macromolecular solute to slide outward and accumulate at the edge of the membrane (where a peripheral polypropylene ring prevents filtration to dryness - deadstop volume =25-40 µl).

Desalting is achieved by concentrating the sample, followed by reconstitution to the original volume using the desired solvent. The process is repeated until the concentration of the contaminating microsolute has been sufficiently reduced. Normally a reduction from 2 ml to 20-40 µl takes one hour (Centricon10) or 30 minutes (Centricon 30).

Concentrate is recovered by capping the sample reservoir with a retenate cup, inverting the device, then centrifuging at 300-1,000 X g.

Retention and Recovery

We were concerned about adsorptive losses because eluant from the gel filtration column contained kinesin in very low concentrations (without column losses, about 4 µg/ml; see Results-Run #5). Since both cytochrome C and kinesin are positively charged, we used cytochrome C (cyt. C) to test for adsorptive loss. According to Amicon, 97.5% of Centricon-10 microconcentrators will recover >85% of cyt. C (0.25 mg/ml). We tried much lower concentrations of cyt. C, and found that untreated Centricon-10 microconcentrators still exhibited about 15% loss whereas units pretreated with Centricon prep. buffer (50 mM Tris, 50 mM KCl, 100 µg/ml BSA) showed 0% loss, presumably because the BSA in solution binds to all nonspecific sites on the membrane.

Procedure

Figure 9: Reference Solutions for Spectrophotometer

[CYT.C] µg/m	A-409
25	.219 ± .004
10	.088 ± .000
7	.064 ± .003
5	.044 ± .001
2.5	.022 ± .000

Centricon 10-untreated: used as supplied by Amicon

Centricon 10-treated: 2 ml of Centricon prep. buffer was concentrated in the microconcentrator. The concentrate was removed by inversion of the sample reservoir followed by centrifugation at 300 - 1,000 X g.

Two ml of test solution containing 21 µg of cyt. C were concentrated with Centricon 10 units (both treated and untreated). The concentrate was resuspended in 3 ml ddH_2O (double-distilled water) which, with 100% recovery, should have produced a solution of 7 µg/ml.

Results

Figure 10: Recovery From Treated and Untreated Centricon 10 Units

	A-409	corresponding [cyt. C]	recovery
UNTREATED	0.053	~ 6 µg/ml	85%
TREATED	0.062	~ 7 µg/ml	100%

Despite the low binding of cytochrome C in BSA-treated Centricon 10 units, a great percentage of bovine k.l.p. in FPLC eluant was lost when attempts were made to concentrate it with Centricon 10 units. We discussed this matter with Bruce Crise, a technician in Bruce Schnapp's lab (The Marine Biological Laboratory, Woods Hole, MA); he confirmed our results, stating that they had experienced up to 90% losses during Centricon 10 concentration of 5-8 ml of 1 mg/ml squid kinesin (much higher concentrations than we were working with). For this reason we began looking for other methods for concentrating bovine k.l.p. in column eluant.

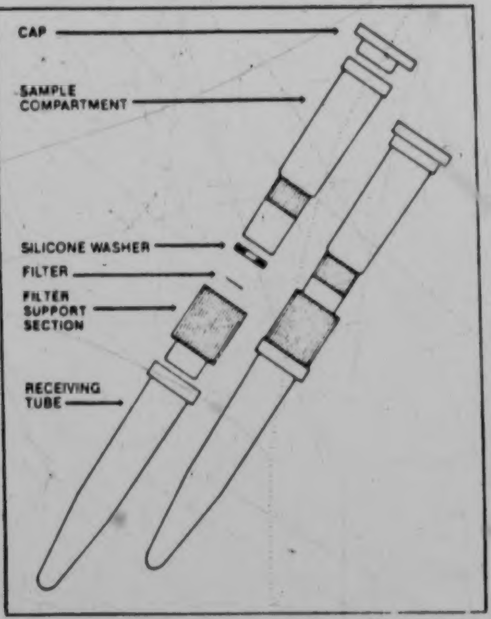
b) Freeze Drying

A volatile buffer (0.1 acetate + ammonium hydroxide pH = 6.4) was used as a column buffer. Fractions were collected, frozen in liquid nitrogen, and exposed to a vacuum (Virtis Freeze-Drying Unit - model 10-103-SD) until total sublimation had occured. The remaining solutes (which were not visible) were resuspended in a small amount of column buffer, treated with Treatment Buffer, and reinjected into the column or run on gels. No proteins were detected by this technique. Perhaps they were lost through adsorption to the polypropylene test tubes used during fraction collection. This technique may be more applicable with solutions containing higher concentrations of protein.

c) Glass Adsorption

Since kinesin was known to adsorb to glass, the following technique was tried as a means of concentrating bovine k.l.p. after gel filtration: about 0.8 g of glass wool was inserted into the sample compartment of a Schleicher and Schuell microfilter apparatus (SS009/0) (see Figure 11) and tamped down with an Eppendorf tip. The unit was then inserted directly into the test-tube receptacles in the FRAC-100 and used to receive a fraction of column eluant. When eluant containing bovine k.l.p. was passed through the glass wool filter, the bovine k.l.p. was trapped. The glass wool was then removed from the microfilter apparatus, placed in a Fisher 1.5 ml micro-centrifuge tube, treated with 20 µl of Treatment Buffer (used for electrophoresis - see next section) and placed in a 100°C water bath for 5 min. The glass wool was then removed from the micro-centrifuge tube, placed back into the microfilter apparatus and centrifuged at 2,000 X g (Servall Angle centrifuge - 1017). When the filtrate was run on PAGE, bovine k.l.p. was found (see Results, Runs #4,5,7,8). This technique was successful, but only yielded denatured bovine k.l.p.. This was adequate for PAGE and immunoblot analysis, but different methods would need to be developed if k.l.p.-generated microtubule or bead motility were an objective.

Figure 11: Schleicher and Schuell Microfilter Apparatus


CAP
SAMPLE COMPARTMENT
SILICONE WASHER
FILTER
FILTER SUPPORT SECTION
RECEIVING TUBE

4. GEL ELECTROPHORESIS

a. Polyacrylamide Gel Electrophoresis

(i) Introduction

When an oligomeric protein is boiled in solution with sodium dodecyl sulfate (SDS) and dithiothreitol (DTT), it dissociates into its subunits; high temperatures cause the weak forces holding the tertiary structure of protein together to be overcome, resulting in a more chaotic, "denatured" state. Each polypeptide chain then becomes coated with a layer of SDS molecules in such a way that the hydrocarbon chains of the SDS molecules are in tight association with the polypeptide chain and the charged sulfate groups of the detergent are exposed to the aqueous medium. Such complexes contain a constant ratio of SDS to protein [about 1.4:1 by weight] and and hence possess a negative charge proportional to length (Lehninger, 1978). When an SDS-treated single chain protein is subjected to an electric field in a molecular-sieve gel containing SDS, its rate of migration is determined primarily by the mass of the SDS-polypeptide particle.

The major usefulness of this system is to determine the molecular weights of polypeptides. A linear relationship exists between log MW of a polypeptide and its R_f (distance from top of gel to polypeptide/ distance from top of gel to dye front). Standard proteins of known molecular weight may be used to generate a standard curve from which the MW of uncharacterized polypeptides (run alongside the standards) may be determined.

The Laemmli system (1970) of SDS-PAGE is a discontinuouos SDS system and probably the most widely used electrophoretic system today. The treated peptides are stacked in a stacking gel before entering the separating gel; therefore, the resolution in a Laemmli gel is excellent.

(ii) Gel Casting and Running

Slab gels were cast using a modified Pharmacia Gel Slab Cassette kit (#II). Two cm plastic spacers with a light coating of glycerin were used in place of the standard spacers, and plasticine was used to seal the bottom of the cassette. These modifications prevented leakage during gel casting. We used the Laemmli (1970) system of SDS PAGE. Acrylamide and N,N'-Methylene-bis-acrylamide from Bio Rad or Schwartz-Mann Biotech were used; little or no difference in gel quality was noted between gels prepared with these two different acrylamide types. Gel solutions were prepared with Tris buffer (pH = 6.8 - stacking gel; pH = 8.8 - separating gel). Monomer stock (30% acrylamide, 2.7% Bis) was added to the gel buffer to make either a 5% or 7.5% (separating gel) or 4% (stacking gel) final concentration. Vacuum was applied to these solutions before sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), and ammonium persulfate were added. The application of vacuum and the careful filtering of all solutions were found to be crucial steps in gel preparation.

Protein containing solutions were put into Fisher 1.5 ml microcentrifuge tubes and treated with 5X Treatment Buffer (0.315 M Tris pH 6.8, 10% SDS, 50% glycerol, 0.25 M DTT) in a ratio of 5:1 (protein solution : 5X Treatment Buffer). The microcentrifuge tubes were then immersed in boiling water for 4 minutes. Solutions were cooled before being applied to gels. Gels were run in a Pharmacia GE 2/4 Electrophoresis Apparatus. Tank buffer (0.025 M Tris pH 8.3, 0.192 M glycerine, 0.1% SDS) was kept between 10-15°C by circulating cooling fluid (Forma Scientific model 2095 refrigeration unit). Power was supplied by a Pharmacia EPS 500/400.

Molecular weight standards were prepared using 10 µl of Bio-Rad SDS-PAGE High molecular weight standards (catalogue #161-0303) which contained 2 mg/ml each of myosin (200 kd), B-galactosidase (116.25 kd) phosphorylase B (92.5 kd), Bovine Serum Albumin (66.2 kd) and ovalubumin (45 kd) in 50% glycerol. This was added to 20 µl of 5X Treatment Buffer + 20 µl ddH$_2$O in a Fisher 1.5 ml microcentrifuge tube and then immersed in boiling water for 4 min. Generally, 5 µl of the final solution (containing 2 µg each of the marking proteins) were applied to gels.

(iii) Gel Staining

After running, the gels were stained with Coomassie blue stain (0.125% Coomassie blue R: Sigma No. B-0630; 50% methanol; 10% acetic acid), which also worked as a fixative. The gels were immersed in the stain overnight, then washed with destaining solution 1 (50% methanol, 10% acetic acid) for two hours followed by destaining solution 11 (7% acetic acid, 5% methanol).

(iv) Gel Scanning

Copmassie blue stained gels were viewed by an RCA video camera (008213). This image was then digitized and stored by a DT-2851 frame grabber (Data Translation). The host computer (an IBM-AT compatible) was used to graph the grey levels of lines chosen by the user, using a program developed by Rolf Manderscheid. These lines were oriented on the lanes in the polyacrylamide gel, so that bands in the polyacrylamide gel appeared as peaks on the monitor. The graph was calibrated with a peak containing a known amount of protein. These graphs were printed on a EPSON FX-80 printer.

b. Non-Denaturing Discontinuous Gels

(i) Introduction

Ekstrom and Kanje (1986) performed ATPase assays of proteins directly upon polyacrylamide gels. We thought this would be an ideal way of correlating ATPase activity with bovine k.l.p.. It was therefore necessary to develop a non-denaturing electrophoretic method for bovine k.l.p., since a standard SDS-PAGE system would destroy the tertiary structure and ATPase activity of sample proteins. Since we had been unable to isolate bovine k.l.p. on IEF agarose gels (see Results-Run #1), a non-denaturing discontinuous buffer PAGE system for cationic proteins at a near-neutral pH was used (Thomas and Hodes, 1981). This system employed a stacking gel which is ideal for focusing dilute samples.

(ii) Gel Casting and Running

The separating gel (9.7% Acrylamide, 0.3% Bis, 0.05 M KOH, 0.05% TEMED, 0.213 M MOPS, 0.0005% Riboflavin, pH = 6.8 - adjusted with acetic acid) was cast using a modified Pharmacia Gel Slab Cassette Kit (#II). Standard 25 W light bulbs were used as a light source for polymerization. After polymerization was completed, stacking gel solution (3.2% Acrylamide, 0.8% Bis, 20.0% glycerol, 0.05 M KOH, 0.1% TEMED, 0.063 M MOPS, 0.0005% Riboflavin 5'-phosphate, pH = 8.0 - adjusted with acetic acid) was added, gel combs were inserted, and light-promoted polymerization was allowed to occur.

Myoglobin and cytochrome C were used as controls. Samples were prepared (no heat treatment) with 1/5 volume of non-denaturing treatment buffer (saturated sucrose + 5% basic fuchsin in ddH_2O). Gels were run in a Pharmacia GE 2/4 Electrophoresis Apparatus. Tank buffer (1.56% Histidine, 0.46% MOPS, 0.46% MOPSO - pH = 6.8, adjusted with acetic acid) was kept between 10-15°C by circulating cooling fluid (Forma Scientific model 2095 refrigeration unit).

Power was supplied by a Pharmacia Electrophoresis Power Supply EPS 500/400. In order to make the bovine k.l.p. enter the gel, the power-supply leads were connected in reverse, so the upper tank buffer was positive and the lower tank buffer was negative.

(iii) Staining

After running, the gels were stained overnight with Coomassie blue stain (0.125% Coomassie blue R: Sigma No. B-0630; 50% methanol; 10% acetic acid) then washed with destaining solution I (50% methanol; 10% acetic acid) for about two hours followed by destaining solution II (7% acetic acid; 5% methanol).

(iv) Results

It became apparent as studies progressed that a non-denatured gel ATPase assay would not work with bovine k.l.p., since studies by Kuznetzov and Gefland (1986) and others (see Introduction, page 21) indicated that the presence of microtubules or high calcium levels were necessary for ATPase activity to occur. High calcium levels could have been produced in the gel, but difficulties encountered in the casting and running of gels limited progress. The proteins did not load well onto the gels, and only diffuse protein bands were produced.

c. Isoelectric Focusing

In isoelectric focusing (IEF), a mixture of proteins is subjected to an electric field in a gel support in which a pH gradient has first been generated. Focusing occurs in two stages; first, the pH gradient is formed. A nonrestrictive gel is polymerized in the presence of highly mobile amphoretic compounds called ampholytes. To generate this gradient, the current is turned on, and the ampholytes arrange themselves according to their isoelectric points, the most acidic moving toward the anode and the most basic toward the cathode. Secondly, each protein then migrates toward, and is "focused" at, that portion of the pH gradient where the pH is equal to its isoelectric pH.

Agarose is a common gel support medium for IEF. It is easy to prepare, chemically inert, optically clear and nontoxic. Agarose has a large pore size which makes it suitable for the focusing of large molecules.

Isoelectric focusing with agarose IEF is most conveniently performed by casting the gel onto a hydrophilic polyester sheet (GelBond: FMC, Marine Colloids Division). We used the procedure outlined in *Isoelectric Focusing: principles and methods* produced by Pharmacia Fine Chemicals. Agarose IEF (Pharmacia) is combined with D-Sorbitol (Sigma) in ddH_2O and melted in a waterbath. Pharmacia Pharmalyte (pH 6.5-9.0) is added and the gel is cast immediately by injecting the liquid agarose between two glass plates (Pharmacia: Agarose IEF kit).

The cooled gel is trimmed and placed on the Pharmacia Flat Bed Apparatus FBE-3000. A cooling system (Forma Scientific Model 2095) keeps the flat bed and gel at 10-15°C. Samples (untreated protein solutions) and standards (Pharmacia Isoelectric Focusing calibration kit, pH 3-10; contains 11 proteins of varying pI) are loaded onto the middle of the gel using paper sample applicators (Pharmacia: Agarose IEF kit). Platinum electrodes are placed directly on the surface of the gel.

A Pharmacia Electrophoresis Constant Power Supply ECPS 3000/150 is then used to run the gel at a constant power setting (usually 6 watts). Voltage increases and amperage decreases as the separation progresses and the resistance of the gel increases. The paper sample dots are removed half way through the run.

After 90 min, the gel is removed from the FBE-3000 and silver stained according to the procedure of Willoughby and Lambert (1983). The gel was fixed for 10 min (3.46 % sulfosalicylic acid, 5% TCA, 5% $ZnSO_4$ in ddH_2O), rinsed in ddH_2O, rinsed in ethanol (95%), pressed between heavy books (protected by filter paper soaked in ethanol) and dried with a hand-held blow drier. It was then rinsed again in ddH_2O, dried, and silver stained for 15-40 min with a combination of 170 ml solution B (1.02 g Ammonium nitrate, 1..02 g Silver nitrate, 5.1 g tungstosilicic acid and 26 ml 10% formaldehyde, brought up to 510 ml with ddH_2O) added to 80 ml solution A (12.0 g Sodium carbonate in 240 ml ddH_2O). The gel is then rinsed briefly in ddH_2O, rinsed for 5 min in 10% acetic acid, and then dried.

5. IMMUNOBLOT

a. Introduction

When a protein is separated in a polyacrilamide gel, it is trapped in the matrix of the gel. The protein can not be detected well by the binding of antibodies because antibodies are too large to diffuse through the gel to bind with specific protein antigens. However, if a protein is transferred from a gel to the surface of a membrane such as nitrocellulose, it becomes easily accessible and extremely sensitive to immunological detection techniques. The fastest method for the transfer of proteins from gels to membranes is electrophoretic transfer. To do this, the gel is simply layered next to the membrane in a gel holding cassette and placed between two electrodes in a tank of transfer buffer. Then a voltage gradient is applied perpendicular to the gel, causing the sample to migrate off the gel and onto the membrane. If SDS-PAGE was used initially to separate the proteins, then the proteins have a negative charge and will migrate towards the anode (positive electrode). After proteins are transferred from the polyacrylamide gel to the nitrocellulose, the membrane is incubated with 3% Gelatin, to block non-specific protein binding sites on the membrane. Antigens are then labelled with specific first antibodies (rabbit origin), located with second antibodies (Biotinylated Goat anti-Rabbit IgG), re-labelled with peroxidase - conjugated Avidin, then identified by HRP-Color Development Solution.

b. Procedure

Protein-containing solutions were separated on an SDS-polyacrylamide gel in the normal fashion. No fixing or staining was performed after electrophoresis.

The transfer tank was built by Dr. Stemke (Dept. of Microbiology, Universtity of Alberta). The cathodic half of the gel cassette was placed on a counter and covered by a moistened foam pad. Moistened blotting paper was placed on top of this foam. The gel was removed from the glass plate sandwich and placed upon the blotting paper. Then an appropriate size of nitrocellulose membrane (Schleicher and Schuell. Pure Nitrocellulose BA-85; 0.45 μm pore size) was moistened and placed directly on the gel, taking care not to trap bubbles beneath. (Note: nitrocellulose should always be handled with gloves). Another piece of moistened blotting paper was placed over the gel, followed by a moistened foam pad and the anodic half of the plexiglass gel cassette. This gave a layered structure as follows: plexiglass (cathodic)/ foam/ blottingpaper/ gel/ nitrocellulose/ blottingpaper/ foam/ plexiglass (anodic).

The cassette was placed into the buffer chamber, and the chamber was filled with buffer (25 mM Tris, 192 mM glycerine, pH = 8.3). The power source (custom-built by Vulgan Electrosystems Ltd. Edmonton; 0-5 Amps, 0-150 volts) was connected to the transfer tank and set at 20 volts - 400 mAmps. The unit was left running in a 4°C cold room overnight.

After electro-blotting, the wet nitrocellulose membrane was immersed in TBS (Tris Buffered Saline: 20 mM Tris, 500 mM NaCl, pH = 7.5) for 10 minutes, then transferred into 37°C Blocking Solution (TBS + 3% Gelatin; Bacto - gelatin : DIFCO Laboratories). The Blocking Solution containing the membrane was gently agitated for one hour on a shaker platform (Thomas Rotating Apparatus No. 3623). The gelatin binds to all protein-binding sites on the membrane which have not been covered by proteins from the electroblot. This prevents nonspecific binding of antibodies to the nitrocellulose.

The membrane was removed from the Blocking Solution and transferred onto a section of Deco-sonic vacuum bag sealer. A Dazey "Seal-a-Meal II" was used to seal three sides of the bag, forming a pocket containing the membrane. Polyclonal Rabbit anti-Squid kinesin (kindly provided by Bruce Schnapp - The Marine Biological Laboratory, Woods Hole, MA) was diluted 1:800 with Antibody Buffer (1% Gelatin - TBS) and approximately 9 ml was poured into the membrane-containing pocket. Air bubbles were removed, the bag was sealed, and the membrane was incubated overnight with gentle agitation (Rocker platform - Bellco Biotechnology).

The membrane was then washed in TBS for 2 X 10 minutes to remove free and nonspecific bound antibody. Washing in TTBS (TBS + 0.05% Tween-20) completely eliminates background staining, but may reduce sensitivity; therefore, TBS washes were generally used.

Biotinylated Goat anti-Rabbit IgG was used as a second antibody. It was prepared by Dr. Stemke's lab as follows: 2 mg of Goat anti-Rabbit IgG in 0.52 ml was added to 0.52 ml of 0.2 M $NaHCO_3$. Then 208 µl of 1 mg/ml BNHS (Nhydroxysiccinimidobiotin) in DMSO (dimethylsulfoxide) was added. This solution was gently agitated for 4 hours at 27°C, then transferred to 1 cm diameter Spectra-por dialysis tubing and dialyzed with 1000 ml TBS overnight at 4°C. The dialysate was then aliquoted, frozen, and stored at -70°C.

This biotinylated Goat anti-Rabbit IgG was diluted 1:300 with Antibody Buffer. Approximately 9 ml was sealed in a bag with the membrane, and gently agitated for 2 hours at 27°C. The membrane was washed again in TBS (2 X 10 min), then incubated for one hour at 27°C with a 1:500 dilution (Antibody Buffer) of peroxidase-conjugated Avidin (Capped-Cooper Biochemical #3000-0470). The membrane was washed in TBS (2 X 10 min) and then immersed into the Development Solution (60 mg Bio-Rad HRP Color Development Reagent [4-chloro-1-napthol] in 20 ml ice cold methanol; added to 60 µl 30% H_2O_2 in 100 ml TBS). Generally, a 5 to 15 minute development period was required to produce easily visible purple bands. The development was stopped by immersing the membrane in distilled water for 10 min.

As a control, rabbit serum (Sigma No. S-2632) was substituted for Rabbit anti-Squid kinesin in the first incubation step.

6. MOTILITY TESTS

We attempted to promote the movement of carboxylated latex beads on microtubules. Samples of Superose 6 purified bovine k.l.p. were collected directly from the column and incubated in various proportions and sequences with 10 mM ATP solution, microtubule-containing solution and latex beads (0.19 µm Polybead-Carboxylate Microspheres from Polysciences). Motility tests were performed in a chamber made from a #1 cover slip (22 X 22 mm) which had been greased around its periphery and a standard microscope slide.

Coverslips were sometimes incubated in poly-d-lysine to examine whether this polypeptide would bind all non-specific sites on the glass surface and allow any bovine k.l.p. present to interact solely with the carboxylated latex beads. The motility chamber was examined using video-enhanced differential interference contrast microscopy. Despite repeated efforts, no motility was seen. This is very possibly due to the low quantities of bovine k.l.p. present in the Superose 6 eluant, which in turn is due to the comparitively low levels of bovine k.l.p. present in our S_6 solutions (see Results, Run #5).

III. RESULTS

A. MICROTUBULE PURIFICATION AND CHARACTERIZATION

In order to carry out microtubule affinity purification of k.l.p. according to the procedure outlined by Vale et al., (1985c), it was necessary to obtain clean microtubules. We used taxol to purify bovine microtubules according to the procedure developed by Vallee (1982) (see Figure 4, page 39). We characterized these microtubules and determined their purity using SDS-PAGE (see Plate I), AVEC-DIC (see Plate II), thin section (see Plate III) and negatively stained (see Plate IV) Transmission Electron Microscopy and Scanning Electron Microscopy (see Plate V). These techniques showed that we had purified 53/56 kd tubulin subunits that polymerized into clean microtubules 20-26 nm in diameter.

We later developed a microtubule-affinity k.l.p. purification technique that utilized endogenous microtubules. This made separate microtubule purification unnecessary and suggested clean, microtubule-associated protein (MAP) free microtubules were not necessary for binding k.l.p.. This is not surprising, since microtubules *in vivo* associate both with MAPs and k.l.p.'s.

PLATE I : SDS Polyacrylamide Gel Electrophoresis of Purified Bovine Brain Microtubules

Gel : 7.5% T, 2.7% C*

Two identical samples of purified microtubules (0.5 µl) were treated with treatment buffer and run on a 7.5% polyacrylamide gel. Only a single doublet may be seen in each lane, indicating the presence of a highly purified protein. The molecular weights of this doublet correspond to the known molecular weights for tubulin subunits (alpha tubulin = 53 kd, beta tubulin = 56 kd). No contaminating proteins are present.

* A 20% T gel would contain 20% w/v of acrylamide plus bis. As the %T increases, the pore size decreases. The other way to adjust pore size is to vary the amount of crosslinker exressed as a percent of the sum of monomer and crosslinker or %C. A 20%T 5%C gel would have 20% w/v of acrylamide plus bis, and the bis would account for 5% of the total weight of the acrylamide. Above and below 5%C, the pore size in the gel increases.

PLATE I

PLATE II : AVEC-DIC of Purified Bovine Brain Microtubules

This photograph shows microtubules reconstituted from the 53/56 kd doublet proteins (see Plate I) and viewed under AVEC-DIC microscopy. Structures shown here are similar to native microtubules viewed by AVEC-DIC in extruded axoplasm preparations of *Homarus americanus* walking-leg axons (B. Maranda, personal communication). In this instance, squid kinesin was incubated with purified bovine brain microtubules on a glass coverslip, which caused the microtubules to bind to the glass surface (see Materials and Methods, page 56).

Actual magnification: 45,000 X

PLATE II

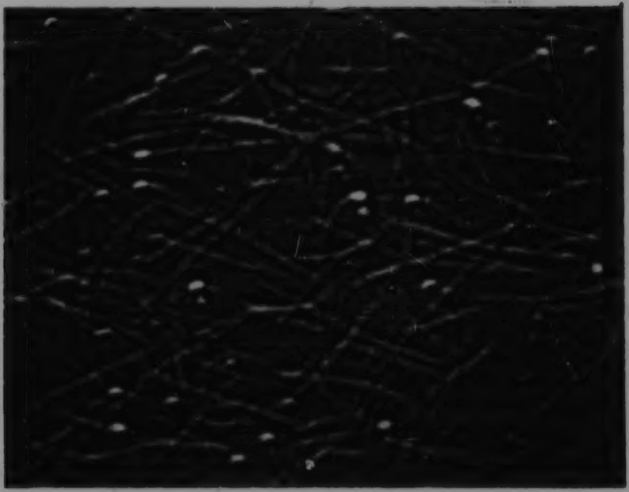


PLATE III : Thin-Section Transmission Electron Microscopy of Purified Bovine Brain Microtubules

Polymeric microtubules can be seen, again showing that the 53/56 kd doublet proteins seen in PAGE are subunits for a larger structure. Cross-sections of the polymeric structure show that the filament is tubular, displaying the hollow center and thin wall characteristic of microtubules.

Actual magnification: 80,000 X

PLATE III

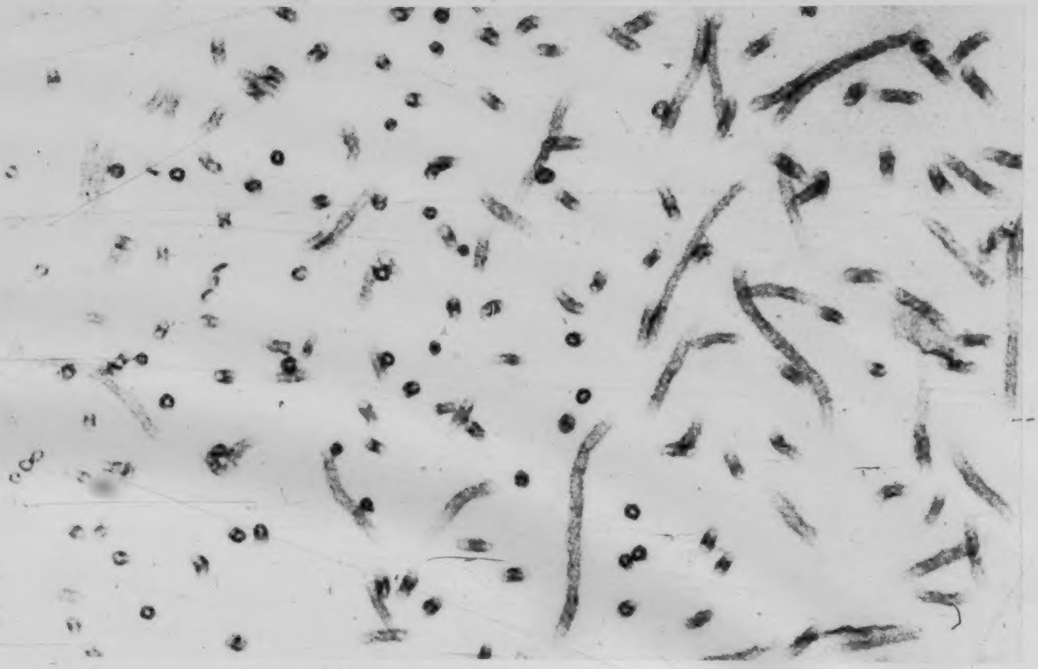


PLATE IV : Transmission Electron Microscopy of Negatively-Stained Purified Bovine Brain Microtubules

These images show that the diameter of the tubule is approximately 20-26 nm, which corresponds to microtubule diameter. Also, the striated appearance of the polymer's surface indicates that the subunits are polymerizing in a regular fashion, forming a coherent substructure of alpha and beta components.

Actual magnification: 200,000 X

PLATE IV

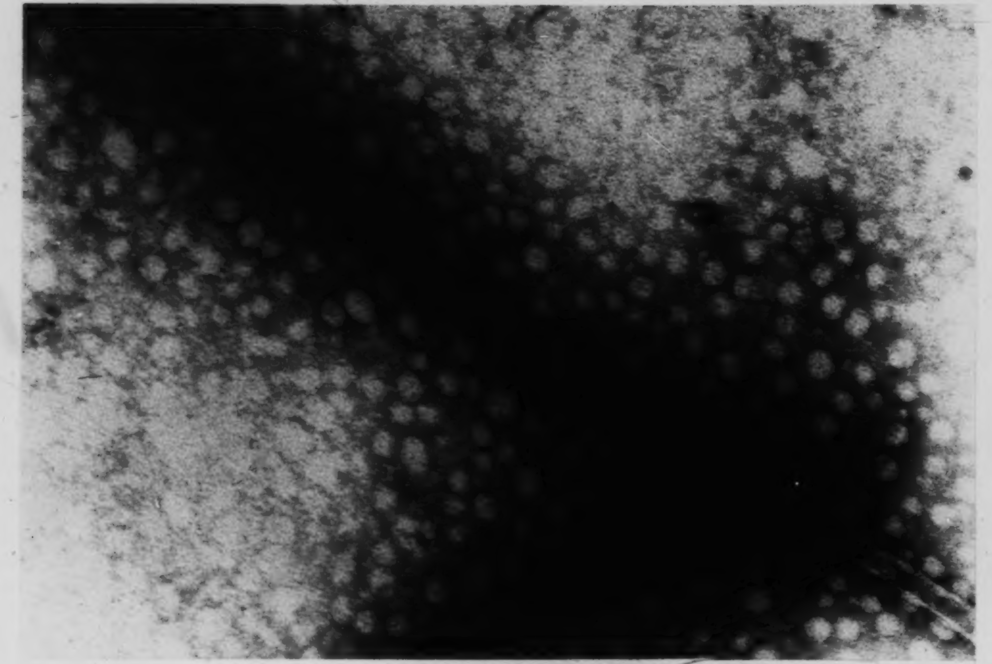


PLATE V : Scanning Electron Microscopy of Purified Bovine Brain Microtubules

This image shows the long filamentous structures of microtubules, showing again that the 53/56 kd doublet proteins seen in PAGE analysis are subunits for a larger polymeric structure.

Actual magnification: 35,000 X

PLATE V

B. K.L.P. PURIFICATION AND CHARACTERIZATION

1. INTRODUCTION

This section will be presented in a chronological fashion. Nine representative "runs" are described. Each run was a separate purification attempt, performed with a specific method and objective. Selected protein-containing solutions from each run were characterized by one or all of IEF, PAGE, gel scans and Superose 6 gel filtration. Immunoblotting, using Rabbit anti-Squid kinesin, was used to positively identify bovine k.l.p..

The following is a collection of selected brief results: A variety of binding agents for the microtubule-affinity purification of k.l.p. were shown to be effective, including tripolyphosphate (PPP), EDTA and AMP-PNP (Run #5). The addition or elimination of wash steps and the alteration of the ratio of crude homogenate (S_1) to purified k.l.p. solution (S_6) were two variations unsuccesfully attempted as a means of increasing the concentration of k.l.p. in the S_6 (Run #2, Run#4). Variations in homogenization buffer showed that the isolation of k.l.p. with AMP-PNP is not critically dependent upon homogenization buffer composition (Run #1). A suspected k.l.p. was isolated from dog brain (Run # 3) (see individual runs for more detailed results).

Negative results have not been included. Using Centricon 10 units or freeze drying for after-column concentration of k.l.p. was unsuccessful, probably due to the high adsorption losses experienced. Non-denaturing PAGE also failed to produce results. However, these results did reveal something about the nature of bovine k.l.p. (see Discussion, page 139). Motility tests and electron microscopy of k.l.p. also failed to produce results; higher concentrations of k.l.p. may be necessary for success with these procedures.

2. NOTATION

In this run and in the notation used throughout these Results, the first number is the run number; 1-S_6* is from the first run, and 4-S_6-PPP is from the fourth run. S refers to supernatant and P refers to pellet, while the subscript number refers to the step in the isolation procedure (see Figures 5 and 6, pages 45 and 53). Notation appearing after the first three symbols refers to specific characteristics of that solution. For example, 1-S_6+, 1-S_6- and 1-S_6* were homogenized with different homogenization buffers. Other solution specific notations include: P, E and PPP, which respectively refer to the use of AMP-PNP, EDTA and tripolyphosphate as binding agents during k.l.p. purification; μ, which indicates that no microtubules were added to the homogenate supernatant during k.l.p. purification; w, which means that an extra wash has been used in the procedure; and cont, (control), indicating that no binding agent was added to the homogenate supernatant during k.l.p. purification.

Solutions to which binding agents were added during purification are often referred to as "experimental" solutions.

When S_6 was run through the Superose 6 column, different fractions relating to different molecular weight ranges were collected and filtered through glass wool. The proteins that adsorbed to the glass were released by heat treatment in Treatment Buffer and characterized by PAGE and immunoblotting (see Materials and Methods, page 63). The nomenclature for these solutions (e.g.: 8-Sup.6-PPP : 500-1000) includes a reference to Sup. 6 and to the fraction's relative molecular weight range in kd.

Please refer to Figures 5 and 6 (pages 45 and 53) for details concerning isolations.

3. RESULTS

a. Run #1 - Experiments with homogenization buffers; isoelectric focusing

It seemed odd to include ATP in the homogenization buffer (Vale et al., 1985c) since its presence would increase the amount of AMP-PNP required to bind k.l.p. to microtubules (see Introduction, page 13). We therefore performed homogenizations of bovine brain using three different homogenization buffers: 1) identical to Vale et al., (1985c); 2) similar to Vale et al., (1985c) but without ATP; 3) simple PEM buffer. Bovine brain k.l.p. was isolated with 5 mM AMP-PNP and clean microtubules. One wash and a 20:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) were used during the purification run (see Figure 5, page 45).

The PAGE gel (Plate VI) shows that the S_3 (high speed homogenate without microtubules) solutions prepared with different homogenization buffers are similar. However, 1-S_6+, 1-S_6- and 1-S_6* (purified k.l.p. solutions, each prepared with different homogenization buffers - see Plate VI) contain extra bands at 120 kd compared to their controls. This is identical to the large subunit size reported by Vale et al., (1985c) for bovine k.l.p., which implies that these buffers can be used successfully to purify bovine k.l.p.. The concentration of the 120 kd protein in experimental 1-S_6 is 24-32 µg/ml (see Figure 12).

PLATE VI : Run #1

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 7.5% T, 2.7% C

Purification Conditions:

lane a : 1-S_6* : homogenization buffer = PEM (0.1 M Pipes, 5.0 mM EGTA, 1.0 mM $MgSO_4$, pH = 6.6); B.A. = 5 mM AMP-PNP

Gel Scan : 120 kd protein - 37.9 µg/ml

lane a': 1-S_6*cont : B.A. = not present

lane b : 1-S_6- : homogenization buffer - as for 1-S_6+, but without 0.5mM ATP; B.A. = 5 mM AMP-PNP

Gel Scan :120 kd protein - 24.8 µg/ml

lane b': 1-S_6-cont : B.A. = not present

lane c : 1-S_6+ : homogenization buffer = 50 mM Pipes, 50 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin,10 µg/ml TAME, 0.5 mM ATP, pH = 7.0

B.A. = 5 mM AMP-PNP

Gel Scan :120 kd protein - 24.2 µg/ml

lane c': 1-S_6+cont : B.A. = not present

For all S_6 : clean microtubules added to 1-S_3, 1 wash, S_1:S_6 = 20:1

lane d = 1-S_3*, lane e = 1-S_3-, lane f = 1-S_3+

Plate VI

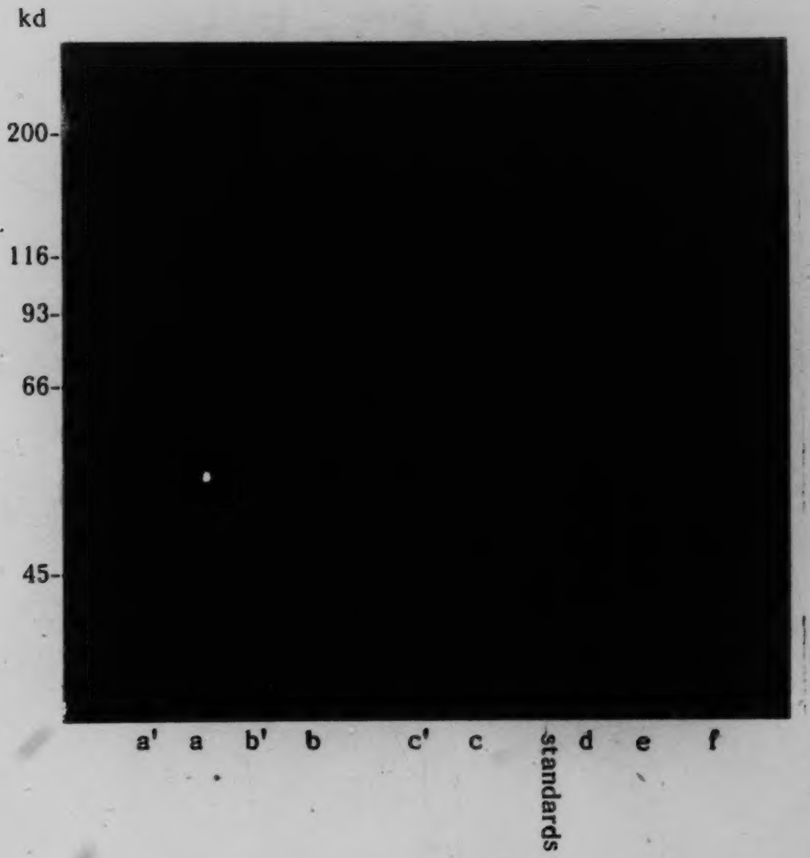

kd
200-
116-
93-
66-
45-
a'
a
b'
b
c'
c
standards
d
e
f

Figure 12 : Run #1

Gel Scanning of Bovine Brain S_6

Figure 12 subheadings:

a: Bio-Rad High Molecular Weight standards

The B-Galactosidase (116.25 kd) band, which corresponds to the peak between the two small rectangles, contained 2 µg of protein. This peak was used to calibrate the scanner.

b: 1-S_{6+} : 120 kd protein - 1.629 µg (from 50 µl sample)

= 32.6 µg/ml

c: 1-S_{6-} : 120 kd protein - 1.312 µg (from 50 µl sample)

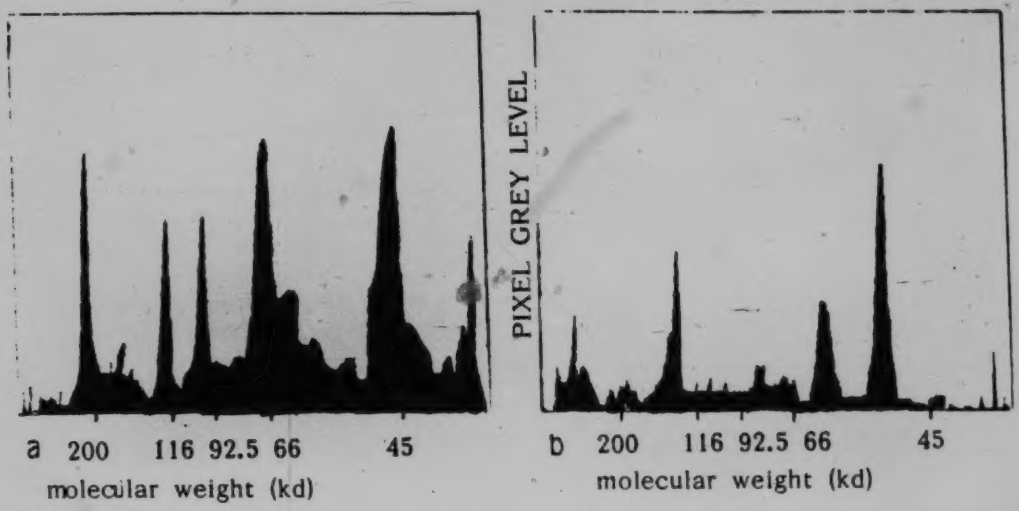
= 26.2 µg/ml

d: 1-$S_{6\bullet}$: 120 kd protein - 2.068 µg (from 50 µl sample)

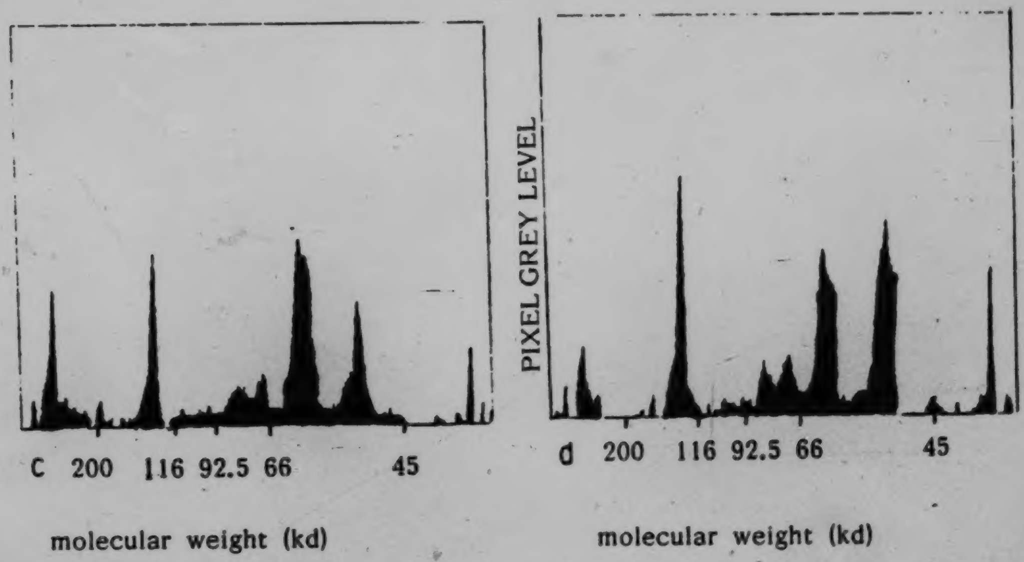
= 41.4 µg/ml

note: gel scans b, c and d corrospond to lanes b, c and d in PlateVI

Figure 12



NOTE: PIXEL GREY LEVEL IS PROPORTIONAL TO THE AMOUNT OF STAINED PROTEIN PRESENT ON THE GEL AT ANY GIVEN MOLECULAR WEIGHT



Experimental 1-S_6 also showed darker bands at 47-48 kd. This protein may be the 43 kd polypeptide which Brady (1985) reported as being significantly increased in the pellet of microtubules incubated with AMP-PNP in chick brain homogenate. The migration of this protein on 2-dimensional gels implied it was actin (Brady, 1985).

When 1-S_{6+} and 1-S_6+cont were run on an isoelectric focusing gel, no additional bands appeared in the experimental solution (see Plate VII). This is despite the results seen with PAGE for these same solutions (see Plate VI). The reason for this is not entirely clear. It is probable that the pH range (3-10) introduced into the agarose gel by ampholytes was not wide enough to accommodate the pI of the k.l.p. This is discussed more fully elsewhere (see Discussion, page 134).

These results show that the isolation of our 120 kd protein is not critically dependent upon homogenization buffer if isolation is performed shortly after homogenization. However, if S_2 or S_3 is frozen in liquid N_2 and kept at -70°C for extended periods of time, solutions prepared without protease inhibitors tend to give lower k.l.p. concentrations when isolations are attempted (results not shown). Therefore, as stated in Materials and Methods, we designed and used our own homogenization buffer.

PLATE VII :Run #1

Isoelectric Focusing of Bovine Brain S_6

No additional bands appear in 1-S_6* which are not present in 1-S_6*cont. This is despite the fact that these two solutions, when run on PAGE (not shown), clearly displayed an additional 120 kd polypeptide present in the 1-S_6* (see Plate VI). This is a representative gel - others showing similar results were run.

lane a : 1-S_6+ : B.A. = 5 mM AMP-PNP

lane a' : 1-S_6+cont : B.A. - not present

For all S_6 : clean microtubules added to 1-S_3; 1 wash; S_1:S_6 = 20:1;
homogenization buffer - as in Run #3

IEF : pH = 3-10
V = 1500 (start)
mA = 151 (start)
W = 6 (constant)
T = 10-12°C

Plate VII

b. Run #2 - EDTA vs AMP-PNP; elimination of wash step

Bovine k.l.p. was isolated with 5 mM AMP-PNP, or 10 mM EDTA and clean microtubules. A 20:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) were used during the purification run. This purification shows that an EDTA-based purification of the 120 kd protein was possible; a gel scan indicated that 2-S_6-P and 2-S_6-E both show a similar concentration increase of a 120 kd protein (30 µg/ml) compared with control (see Plate VIII).

Also, the wash step (S_4-S_5) was eliminated (see Figure 5, page 45); the elimination of this step did not increase the concentration of kinesin in the experimental S_6 The levels of contaminating proteins that remained loosely associated with the unwashed pellet were much higher (compare to Plate VI); the presence of additional microtubule-binding proteins that are normally removed by the wash is also possible. This procedure was not continued.

PLATE VIII : Run #2

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 7.5% T, 2.7% C

Purification Conditions:

lane a : 2-S_6-P : B.A. = 5 mM AMP-PNP

Gel Scan : 129 kd protein - 30 µg/ml

lane b : 2-S_6cont : B.A. = not present

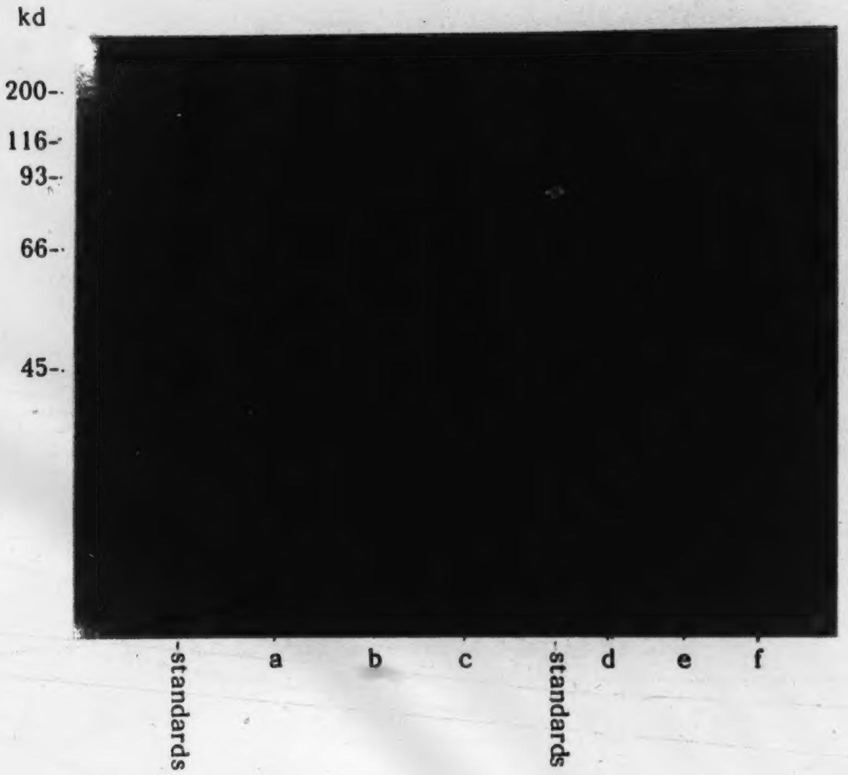
lane c : 2-S_6-E : B.A. = 10 mM EDTA

Gel Scan : 120 kd protein - 30 µg/ml

For all S_6 : clean microtubules added to 2-S_3; no wash; homogenization buffer - as for 1-S_6+; S_1:S_6 = 20:1

lane d = 2-P_6-P, lane e = 2-P_6cont, lane f = 2-P_6-E

Plate VIII


kd
200-
116-
93-
66-
45-
-standards
a
b
c
-standards
d
e
f

c. Run #3 - Dog Brain

We thought it would be interesting to purify a k.l.p. from an alternate tissue, so we obtained brains from dogs used in experimental surgery classes at the S.M.R.I. (see Materials and Methods, page 50). EDTA was again used as a binding agent in this purification, but a range of concentrations from 40 to 100 mM were used to determine ideal concentration (note: a previous run had suggested that EDTA concentrations up to 40 mM would be appropriate - results not shown). The increase of EDTA concentration over 40 mM did not increase the concentration of proteins of interest in 3-S_6-E, so we standardized 40 mM as being the ideal EDTA concentration for our isolations. The extra bands for this suspected canine k.l.p. seem to be slightly lower (about 116 kd) than the bands seen for bovine k.l.p. and are composed of doublets rather than singlets (see Plate IX). Doublets were also occasionally seen for bovine k.l.p. (see Results - Run #4).

We also attempted, unsuccessfully, to use another control. The addition of microtubules at the S_3 incubation step was eliminated for 3-S_3-Eμ and 3-S_3-Pμ, purportedly to show the extra proteins in experimental S_6 solutions depended upon microtubules for their purification (see Figure 5, page 45). Paradoxically, the 3-S_6-Pμ showed the strongest bands both for the 116 kd doublet (66 μg/ml) and for microtubules, and the 3-S_6-Eμ also showed the presence of microtubules. Obviously not all microtubules had been removed during the S_2 to S_3 step. The control solution which was incubated with microtubules but no EDTA or AMP-PNP (3-S_6cont) also showed the presence of a 116 kd doublet, which could have been due to the absence of ATP in the homogenization buffer (ATP depletion induces a strong binding state between kinesin and microtubules - Vale et al., 1985b). This possibility is discussed more fully elsewhere(see Discussion, page144).

PLATE IX : Run #3

SDS Polyacrylamide Gel Electrophoresis of Dog Brain S_6

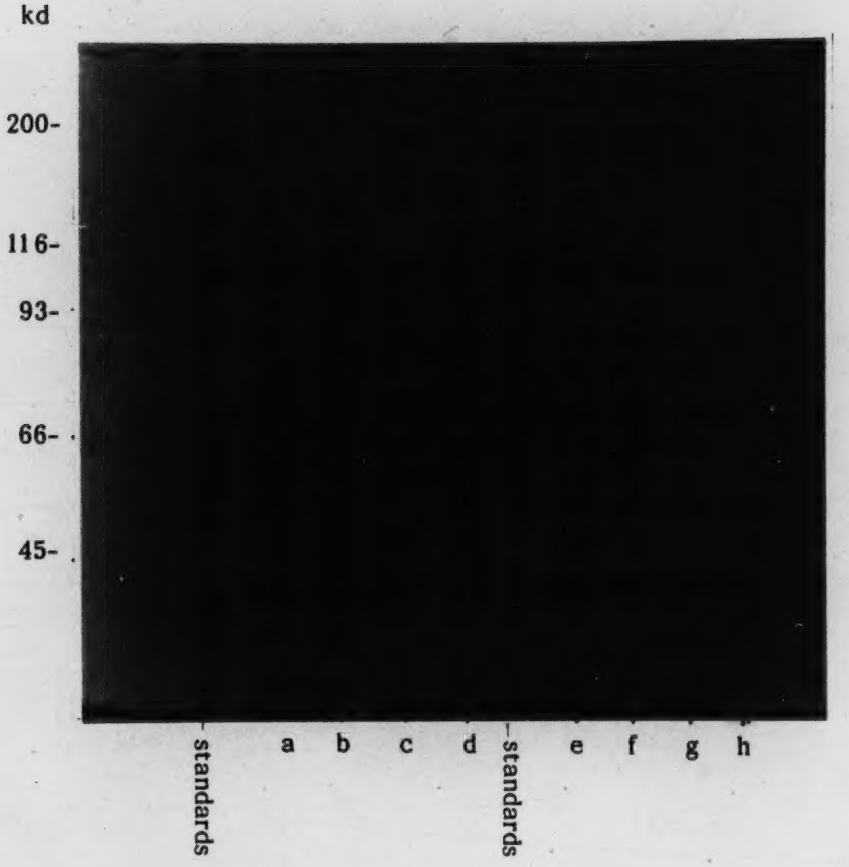
Gel : 7.5% T, 2.7% C

Purification Conditions:

lane a :	3-S_6-P	: B.A. = 5 mM AMP-PNP; clean microtubules
lane b :	3-S_6-Pμ	: B.A. = 5 mM AMP-PNP; no microtubules Gel Scan : 116 kd protein - 66.0 μg/ml
lane c :	3-S_6-Eμ	: B.A. = 40 mM EDTA; no microtubules
lane d :	3-S_6cont	: B.A. - not present; clean microtubules
lanes e, f, g, h :	3-S_6-E-(40, 60, 80, 100):	B.A. = EDTA (40 mM, 60 mM, 80 mM, 100 mM); clean microtubules

For all S_6 :1 wash; S_1:S_6 = 20:1; homogenization buffer = 0.1 M Pipes, 5 mM EGTA,1.0 mM $MgCl_2$, 1.0 mM DTT, 5.0 μg/ml leupeptin, 5.0 μg/ml TAME, 0.1 mg/ml soybean trypsin inhibitor; "clean microtubules" means that clean microtubules were added to 3-S3 for that particular solution.

Plate IX


kd
200-
116-
93-
66-
45-
standards
a
b
c
d
standards
e
f
g
h

d. Run #4 - Use of PPP; extra wash; glass wool technique

With this run we first showed that our suspected bovine brain k.l.p. could be isolated by using 5 mM PPP as a binding agent. This idea was taken from Kuznetsov and Gefland (1986). Clean microtubules and a 20:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) were used during the purification run. An extra wash was tried between steps 5 and 6 (see Figure 5, page 45) for the 4-S_6-PPPw solution. This did not make it much cleaner than the 4-S_6-PPP, so the use of an extra wash step was not standardized.

In this experiment, 0.2 ml of 4-S_6-PPP (containing 2 µg of bovine k.l.p.-see Plate X) was injected into a Superose 6 column. Fractions were collected and filtered through glass wool. The glass wool was then heat treated with Treatment Buffer (see Materials and Methods, page 63) and centrifuged. Released proteins were analyzed by PAGE. The fraction corresponding to 500-1,000 kd (which is within the range reported for kinesin and k.l.p.'s - see Figure 2, page 18) contained a 120 kd band. This band contained approximately 0.33 µg of protein, corresponding to 16.5% of the bovine k.l.p. injected onto the column. It was unclear whether the 83.5% loss was due to inefficient binding to the glass wool or due to losses within the column.

This was the first connection made between the 120 kd subunit and a larger 500-1,000 kd molecule. It is interesting to note that this isolation shows a doublet rather than a single band at 120 kd. It may be that subtle differences in gel casting or running technique result in a finer focusing of our suspected bovine k.l.p. into a doublet.

PLATE X : Run #4

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 7.5% T, 2.7% C

Purification Conditions:

lane d : 4-S_6cont : 1 wash

lane e : 4-S_6- PPP : B.A. = 5 mM PPP,1 wash
Gel Scan : 120 kd protein - 10 µg/ml

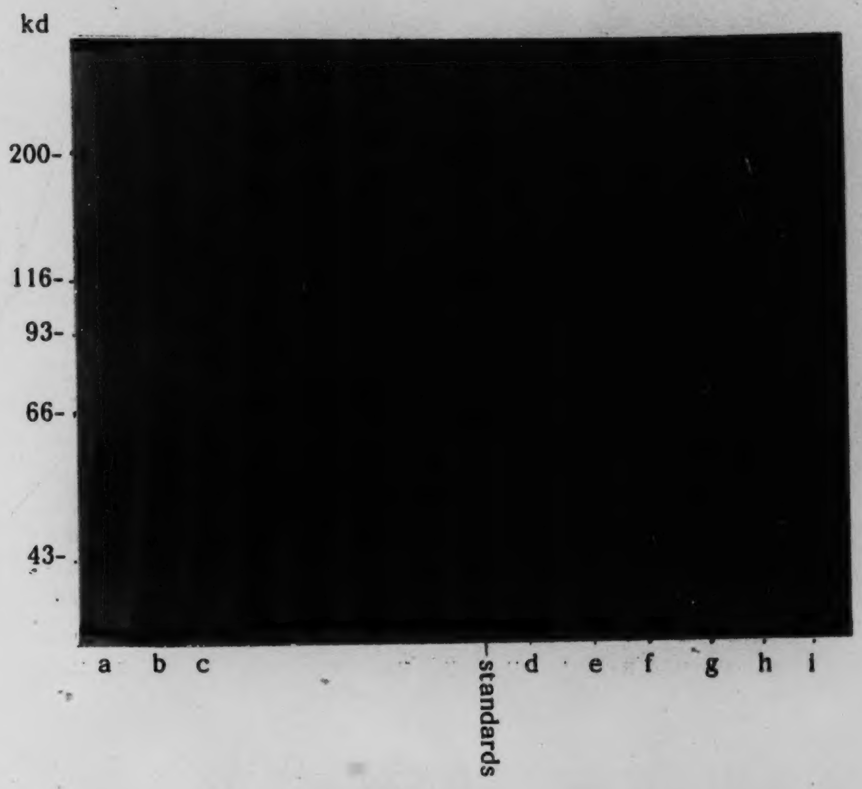
lane f : 4-S_6-PPPw : B.A. = 5 mM PPP, 2 washes
Gel Scan : 120 kd protein - 12 µg/ml

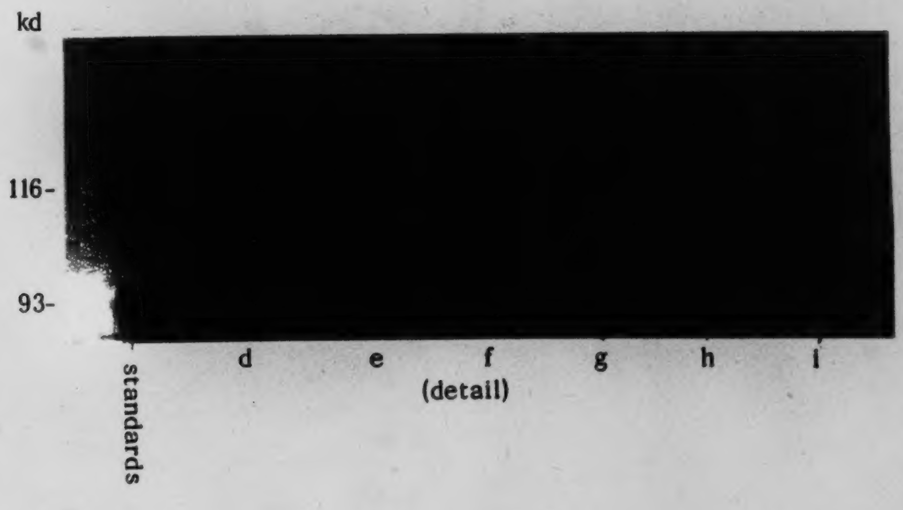
For all S_6: clean microtubules added to 4-S_3; S_1:S_6 = 20:1;
homogenization buffer - as in Run #3

FPLC: Superose 6 column; buffer = 0.05 M Tris, 0.1 M KCl, 5 mM $MgCl_2$, pH = 7.6; 200 µl each of 4-S_6-PPP and 4-S_6cont injected onto column; fractions collected by FRAC 100; glass wool eluant filtration used.

lane a : 4-Sup. 6cont : >1,000 kd
lane b : 4-Sup. 6cont : 500-1,000 kd
lane c : 4-Sup. 6cont : <500 kd
lane g : 4-Sup.6-PPP : >1,000 kd
lane h : 4-Sup.6-PPP : 500-1,000 kd;Gel scan:120 kd protein - 0.33µg
lane i : 4-Sup.6-PPP :<500 kd

Plate X


kd
200-
116-
93-
66-
43-
a
b
c
standards
d
e
f
g
h
i


kd
116-
93-
standards
d
e
f
g
h
i
(detail)

e. Run #5 - Immunoblotting 5-S_6; pinpointing k.l.p. losses

In this isolation, AMP-PNP worked much better than EDTA or PPP in promoting the purification of bovine k.l.p. (30.4, 7.2 and 9.2 µg/ml for 5-S_6-P, 5-S_6-PPP and 5-S_6-E respectively - see Plate XI). In that sense, these are unusual results. However, this was the first run where immunoblotting was used to identify bovine k.l.p. (see Plate XII); both 5-S_6-PPP and 5-S_6-P showed definite labelling of the 120 kd polypeptide by Rabbit anti-Squid kinesin. Shared antigenicity with squid kinesin and shared large subunit molecular weight with bovine k.l.p. (Vale et al., 1985c) were sufficient evidence to allow the labelling of this 120 kd protein as bovine k.l.p.

This was also the first run where the S_1:S_6 ratio was altered in an attempt to increase the S_6 concentration of bovine k.l.p.. A ratio of 40:1 was used. However the highest concentrations of bovine k.l.p. we were able to achieve were still an order of magnitude less than the S_6 kinesin concentrations of 1 mg/ml claimed by Vale et al., (1985c). Possible reasons for this discrepancy are discussed elsewhere (see Discussion, page 142).

This run also allowed us to localize our bovine k.l.p. losses to the Superose 6 column. This was accomplished by correlating FPLC and PAGE results as follows: PAGE analysis showed that 30.4 µg/ml of the 112 kd subunit of bovine k.l.p. was present in 5-S_6-P. If other possible subunit contributions are ignored, this can also be taken as the concentration of bovine k.l.p. in 5-S_6-P. Since 0.2 ml of 5-S_6-P (containing 6.08 µg bovine k.l.p.) was injected into the column, and only a 2% peak (roughly corresponding to 0.25 µg - see Figure 13) was detected for bovine k.l.p. after the column, 96% of the bovine k.l.p. was being lost during gel filtration. The glass walls of the separating column were deemed to be the problem in this case (Vale et al., 1985c).

PLATE XI : Run #5

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 7.5% T, 2.7% C

Purification Conditions:

lane e : 5-S_6cont : B.A. = not present

lane f : 5-S_6-P : B.A. = 5 mM AMP-PNP

Gel Scan : 120 kd protein - 30.4 µg/ml

lane g : 5-S_6-PPP : B.A. = 5 mM PPP

Gel Scan : 120 kd protein - 9.2 µg/ml

lane h : 5-S_6-E : B.A. = 40 mM EDTA

Gel Scan : 120 kd protein - 7.2 µg/ml

For all S_6 : clean microtubules added to 5-S_3; 1 wash; S_1:S_6 = 40:1; homogenization buffer - as in Run #3

FPLC: Superose 6 column; buffer = 0.05 M Tris, 0.1 M KCl, 5 mM $MgCl_2$, pH = 7.6; 200 µl samples of 5-S_6-P, 5-S_6-PPP and 5-S_6-E injected; fractions collected by FRAC 100; glass wool eluant filtration used.

lane a : 5-Sup.6cont: >1,000 kd

lane b : 5-Sup.6cont: 500-1,000 kd

lane c : 5-Sup.6-P :>1,000 kd

lane d : 5-Sup.6-P : 500-1,000 kd; Gel Scan:120 kd protein - 0.20µg

lane i : 5-Sup.6-PPP: >1,000 kd

lane j : 5-Sup.6-PPP: 500-1,000 kd; Gel Scan:120 kd protein - 0.05µg

lane k : 5-Sup.6-E : >1,000 kd

lane l : 5-Sup.6-E : 500-1,000 kd

Plate XI

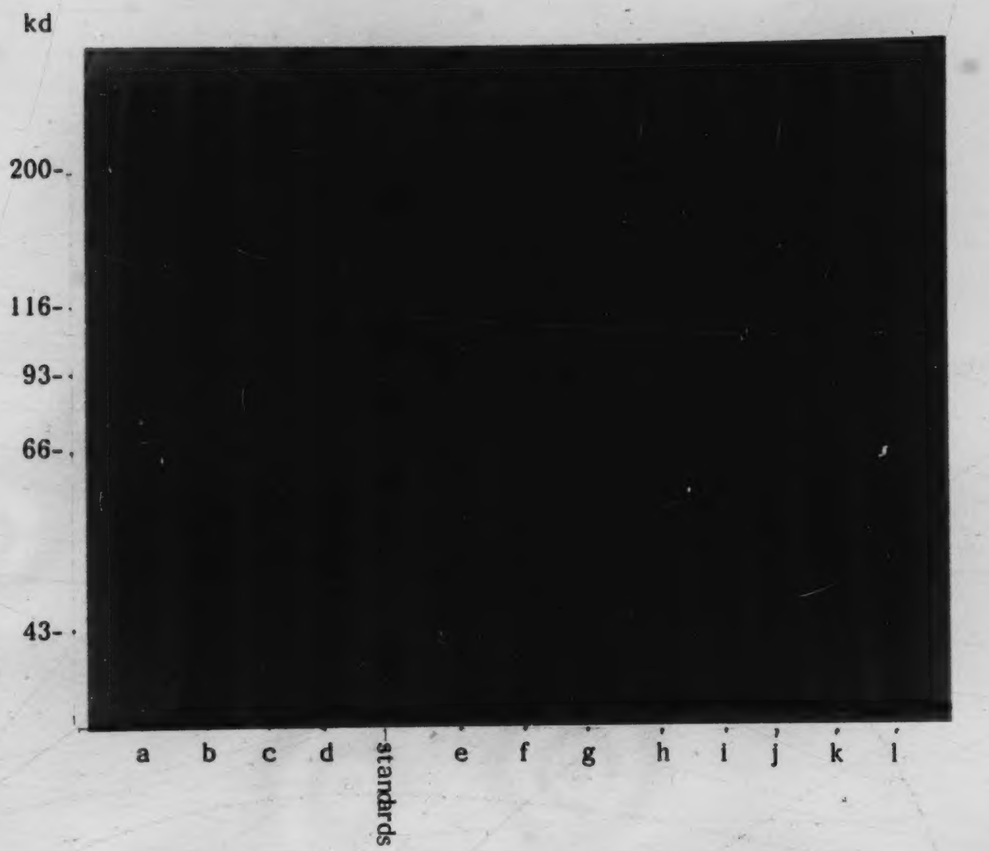

kd
200-
116-
93-
66-
43-
a
b
c
d
standards
e
f
g
h
i
j
k
l

PLATE XII : Run #5

Immunoblotting of Bovine Brain S_6

Standard procedure for immunoblotting was used, except no normal rabbit serum (n.r.s) control was run.

lane a : 5-S_6-E

lane b : 5-S_6-PPP

lane c : 5-S_6-P

lane d : 5-S_6cont

Rabbit anti-Squid kinesin identified subunit
in 5-S_6-P and 5-S_6-PPP

The bovine k.l.p. bands identified by immunoblot faded over time; these results do not have their original clarity. Also, no standards were run on the gel used for the immunoblot, so no direct molecular weight markers can be shown in the picture. However, the gel in Plate X was cast and run at the same time as the gel used in the immunoblot, so the standards in this gel were used to characterize the immunoblot.

Plate XII

Figure 13 : Run #5

Superose 6 Gel Filtration of Bovine Brain S_6

Buffer : 0.1 M KCl

0.05 M Tris

5.0 mM $MgCl_2$

pH = 7.6

Abs. : 280

Sensitivity : 0.05 (full scale deflection corrosponds to an absorbance of 0.05)

ml/min : 0.4

cm/ml : 0.5

note : Pharmacia REC 482 charts have been reduced 2.4 X . Each division along the X axis corrosponds to 2 ml eluant

Figure 13 subheadings:

a: 5-S_6cont

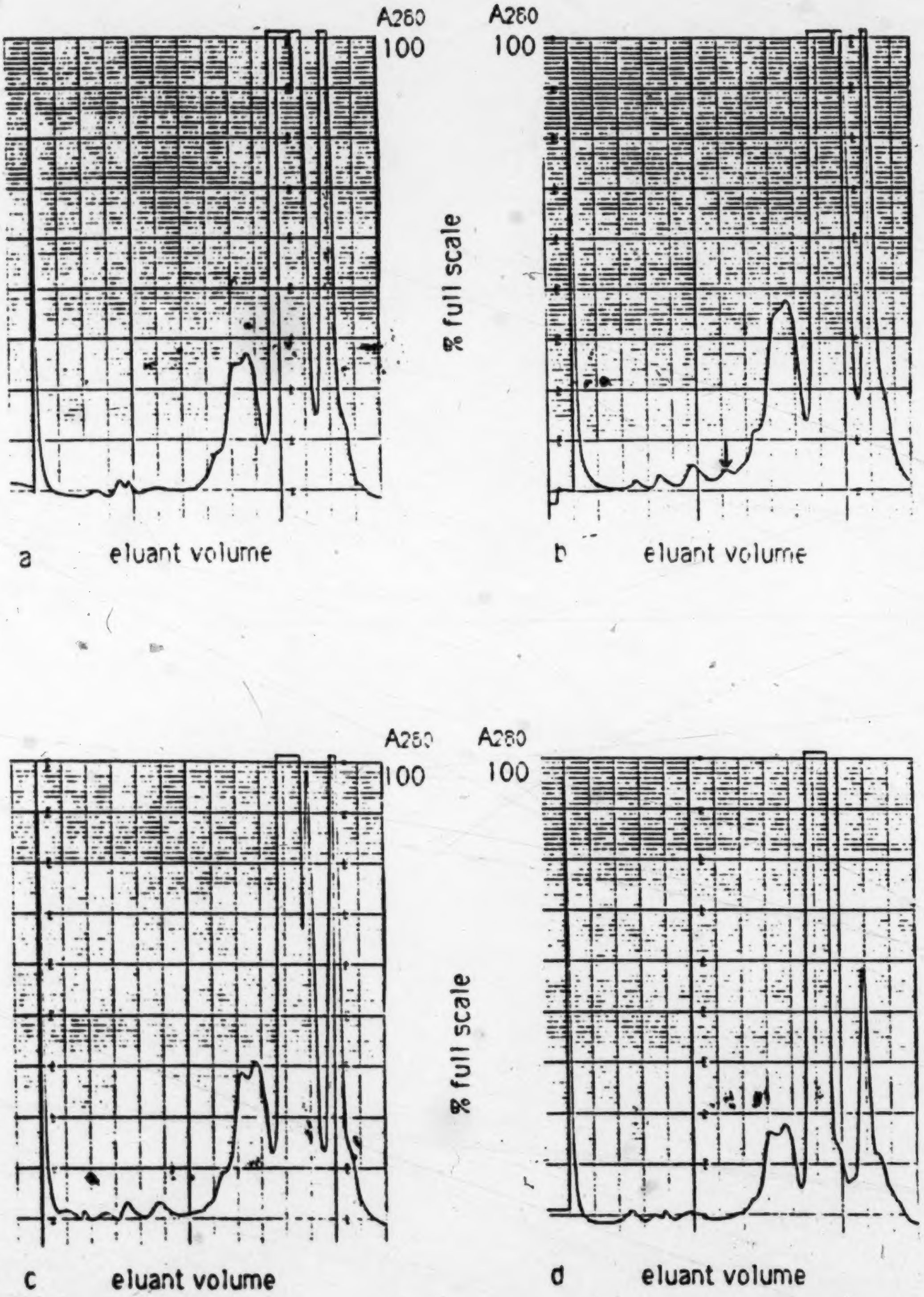
b: 5-S_6-P

c: 5-S_6-PPP

d: 5-S_6-E

For each: column eluant collected with glass wool filtration; fractions collected from 10-14 ml (500-1,000 kd, see Figure 7, page 62) contained bovine k.l.p. (see Plate X : 5-Sup.6-PPP : 500-1000, 5-Sup.6-P : 500- 1000). 5-S_6-P showed a small 2% peak in this region on the REC 482 chart (Figure 13b; see arrow).

Figure 13:


A260
100
A260
100
% full scale
a
eluant volume
b
eluant volume
A260
100
A260
100
% full scale
c
eluant volume
d
eluant volume

The glass filtration method of after-column bovine k.l.p. recovery gave a band of approximately 0.2 µg at 120 kd (see Plate XI). This shows that k.l.p. recovery by glass filtration is close to 100%, and suggests that the losses seen in Run #4 were due to column losses rather than to inefficient glass binding.

f. Run #6 - Squid Kinesin; ATP-containing column buffer

In this run, bovine brain k.l.p. was isolated with 40 mM EDTA and 5 mM PPP as binding agents. Clean microtubules, one wash and a 40:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) were used during the purification run. PPP and EDTA promoted approximately equal levels of bovine k.l.p. binding (26 vs 30 µg/ml). Squid kinesin (supplied by Bruce Schnapp, The Marine Biological Laboratory, Woods Hole, MA) was run beside 6-S_6-PPP and 6-S_6-E on PAGE (see Plate XIII). This showed that the large subunit for squid kinesin has a slightly lower molecular weight than the 120 kd bovine k.l.p.. This parallels results reported by Vale et al., (1985c).

The squid kinesin was also run parallel to bovine k.l.p.-containing solutions for an immunoblot. As expected, the Rabbit anti-Squid kinesin bound better to squid kinesin than to bovine k.l.p. (see Plate XIV). The lower squid subunit size is also seen in these results.

For the first time we tried to reduce column losses of bovine k.l.p. by changing the column buffer. Following the procedure of Vale et al. (1985c) we added ATP and EDTA in the column buffer, though no rationalization could be seen for using these agents (EDTA would act against the Mg^{++} ions in the same buffer, and ATP, it is believed, does not act on the glass-binding area of bovine k.l.p. - Vale et al. 1985c). No reductions in column losses of bovine k.l.p. were seen (results not shown). The extremely high background absorption caused by the presence of ATP in this buffer made sensitive detection of low amounts of bovine k.l.p. impossible.

PLATE XIII : Run #6

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6 and Squid Kinesin

Gel : 7.5% T, 2.7% C

Purification Conditions (Bovine Brain):

lane a : 6-S_6cont : B.A. - not present

lane b : 6-S_6-PPP : B.A. = 5 mM PPP
Gel Scan : 120 kd protein - 26 µg/ml

lane c : 6-S_6-E : B.A. = 40 mM EDTA
Gel Scan : 120 kd protein - 30 µg/ml

lane d : squid kinesin
Gel scan : 116 kd protein - 66 µg/ml

For all S_6 : clean microtubules added to 6-S_3; 1 wash; S_1:S_6 = 40:1; homogenization buffer - as in Run #3

Plate XIII

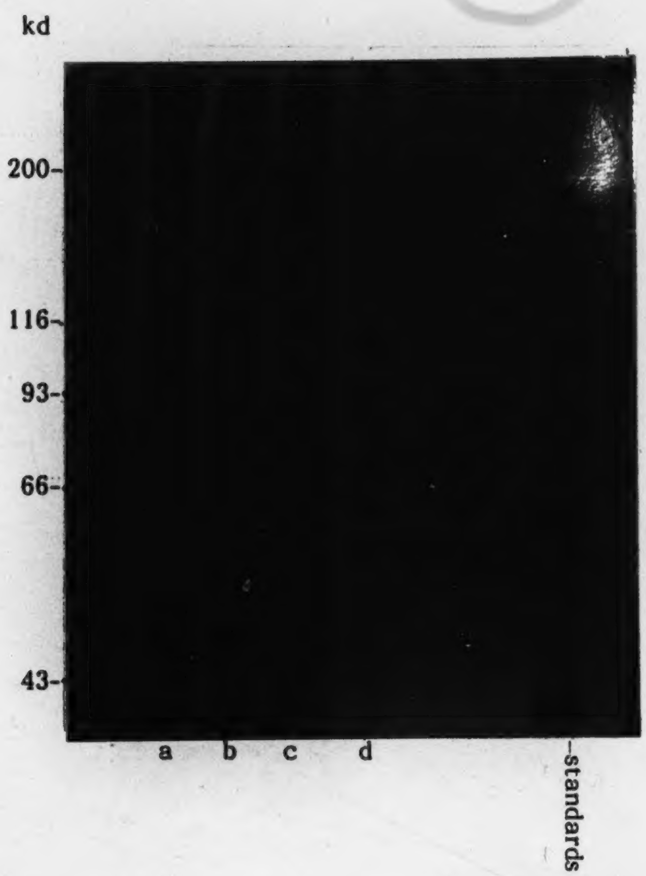

kd
200-
116-
93-
66-
43-
a
b
c
d
-standards

PLATE XIV : Run #6

Immunoblotting of Squid Kinesin

Standard procedure for immunoblotting was used, except no normal rabbit serum (n.r.s.) control was run

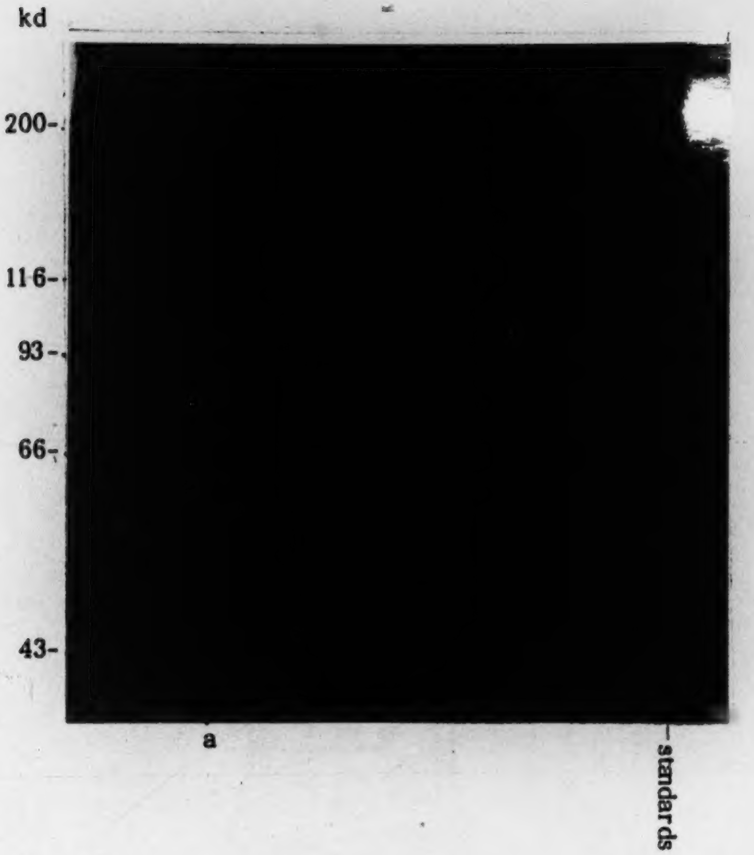
Rabbit anti-Squid kinesin strongly identified 116 kd subunit of squid kinesin.

Other lanes show various bovine k.l.p.-containing solutions (from left to right: blank, 6-S_2, 6-S_6-E, 6-S_6cont, 6-S_6-PPP)

The Rabbit anti-Squid kinesin antibodies are more reactive with squid kinesin than with bovine k.l.p. (see Plate XIII).

The portion of the polyacrylamide gel containing the molecular weight standards was cut off and stained in Coomassie blue. Immunoblotting was performed on the rest of the gel.

Plate XIV


kd
200-
116-
93-
66-
43-
a
standards

g. Run #7 - Endogenous microtubules; immunoblotting 7-S_2

In this run, bovine brain k.l.p. was bound to endogenous microtubules with 40 mM EDTA and 10 mM PPP. A 40:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) was used during the purification run. The fact that endogenous microtubules could be used in the purification of k.l.p. (see Plate XV) indicated that the presence of MAPs did not prevent the binding of bovine k.l.p. to microtubules. Though the S_6 may have been slightly dirtier compared to earlier runs performed with clean microtubules, the added simplicity made this procedure very attractive. Also, it was possible that an ATP-depletion condition existed in our S_2, since no ATP was included in our homogenization buffer. This could have caused bovine k.l.p. to bind to endogenous microtubules which were being discarded in previous runs. However, it is unlikely that much bovine k.l.p. was being lost in this way, since S_6 bovine k.l.p. yields for Run #7 and Run #8 are similar to earlier runs.

The immunoblot (Plate XVI) showed that Rabbit anti-Squid kinesin could pick bovine k.l.p. out of all the proteins present in 7-S_2 (compare this to the 7-S_2 on Plate XV). Excellent immunoblot results were also obtained for 7-S_6-PPP and 7-S_6-E.

Although the ATP and EDTA added to the column buffer did not reduce column losses (over 90% still disappeared during gel filtration), the glass-wool technique again showed that bovine k.l.p. present in 7-S_6-E and 7-S_6-PPP eluted from the column at a position corresponding to 500-1,000 kd. The FPLC charts are not shown in this case, since the presence of ATP in the buffer created a very high baseline that made them unreadable.

PLATE XV : Run #7

SDS Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 7.5% T, 2.7% C

Purification Conditions:

lane f : 7-S_6cont : B.A. - not present

lane g : 7-S_6-PPP : B.A. = 10 mM PPP
Gel Scan : 120 kd protein - 10 µg/ml

lane h : 7-S_6-E : B.A. = 40 mM EDTA
Gel Scan : 120 kd protein - 10 µg/ml

For all S_6 : endogenous microtubules used in 7-S_3; 1 wash; S_1:S_6 =40:1; homogenization buffer - as in Run #3

FPLC: Superose 6 column; Buffer = 0.05 M Tris, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH = 7.6; 200 µl each of 7-S_6-PPP and 7-S_6-E injected onto column; fractions collected by FRAC 100; glass wool eluant filtration used.

lane a : 7-S_2
lane b : 7-Sup.6-PPP : >1,000
lane c : 7-Sup.6-PPP : 500-1,000; Gel scan: 120 kd protein - 0.15 µg
lane d : 7-Sup.6-E : >1,000
lane e : 7-Sup.6-E : 500-1,000; Gel scan: 120 kd protein - 0.20 µg

Plate XV

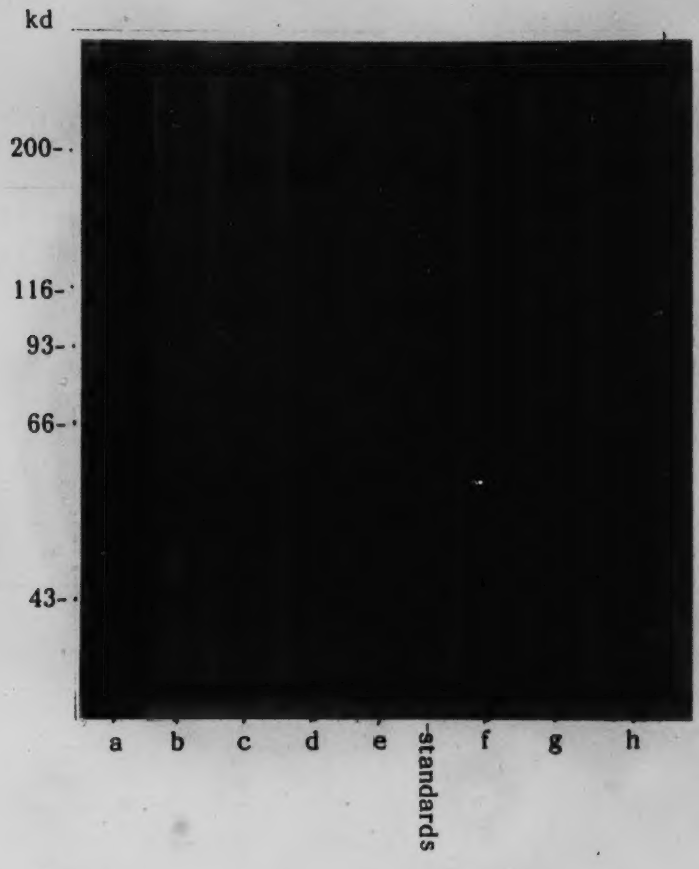

kd
200-
116-
93-
66-
43-
a
b
c
d
e
standards
f
g
h

PLATE XVI : Run #7

Immunoblotting of Bovine Brain S_6 and S_2

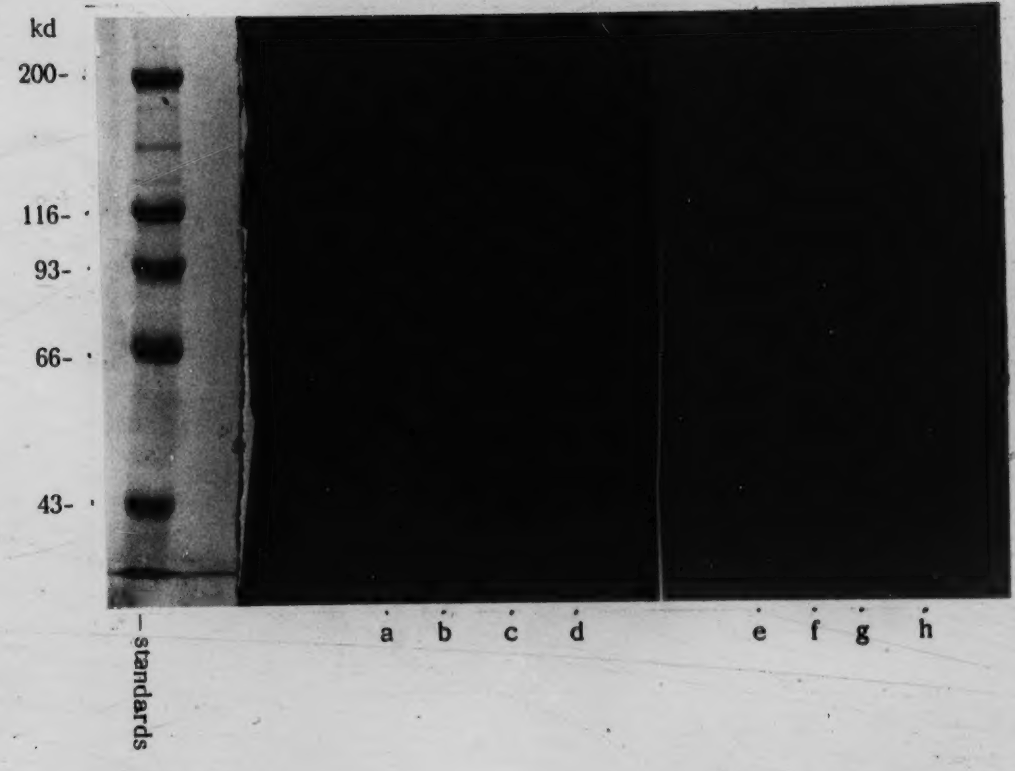
Standard procedure

normal rabbit serum (n.r.s.) control used

lane a : 7-S_2 (n.r.s. control)
lane b : 7-S_6-E (n.r.s. control)
lane c : 7-S_6-PPP (n.r.s. control)
lane d : 7-S_6cont (n.r.s. control)
lane e : 7-S_2 (contained bovine k.l.p.)
lane f : 7-S_6-E (contained bovine k.l.p.)
lane g : 7-S_6-PPP (contained bovine k.l.p.)
lane h : 7-S_6cont (no bovine k.l.p. detected)

It is interesting to note the wider separation between the doublet for bovine k.l.p. when it is run as 7-S_2 rather than 7-S_6. This could be due to the presence of large amounts of protein in 7-S_2 which could alter the migration of individual proteins in the gel.

Plate XVI


kd
200-
116-
93-
66-
43-
-standards
a
b
c
d
e
f
g
h

h. Run #8 - Immunoblotting FPLC eluant

In this run, bovine brain k.l.p. was bound to endogenous microtubules with 40 mM EDTA and 10 mM PPP. A 60:1 ratio between S_1 (crude homogenate) and S_6 (purified k.l.p. solution) was used during the purification run in an attempt to increase the concentration of bovine k.l.p. in S_6 (8-S_6-E: 30 µg/ml; 8-S_6-PPP: 40 µg/ml - see Plate XVII).

ATP and EDTA were removed from the column buffer. A small peak corresponding to the expected location for bovine k.l.p. could be discerned for 8-S_6-PPP (see Figure 14). Immunoblotting allowed the qualitative but not quantitative identification of bovine k.l.p. in the fraction from 8-S_6-PPP corresponding to 500-1,000 kd (see Plate XVII). This was the only immunological identification of glass-filter collected column-purified bovine k.l.p. achieved; 8-Sup.6-E : 500 - 1000 did not blot, but the amount of evidence necessary for achieving conclusions for this thesis was considered to be more than adequate.

PLATE XVII : Run #8

Polyacrylamide Gel Electrophoresis of Bovine Brain S_6

Gel : 5% T, 1.4% C

Purification Conditions:

lane a : 8-S_6cont : B.A. - not present

lane b : 8-S_6-E : B.A. = 40 mM EDTA
Gel Scan : 120 kd protein - 30 µg/ml

lane c : 8-S_6-PPP : B.A. = 10 mM PPP
Gel Scan : 120 kd protein - 40 µg/ml

lane d : 8-S_3

For all S_6: endogenous microtubules used in 8-S_3; 1 wash; S_1:S_6 = 60:1;
homogenization buffer - as in Run #3

Plate XVII

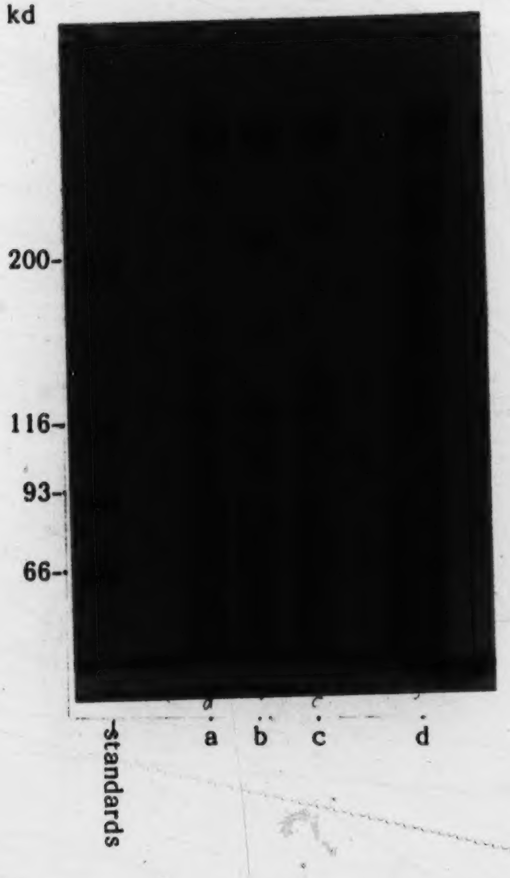

kd
200-
116-
93-
66-
standards
a
b
c
d

Figure 14 : Run #8

Superose 6 Gel Filtration of Bovine Brain S_6

Buffer : 0.1 M KCl
0.05 M Tris
5.0 mM $MgCl_2$
pH = 7.6

Abs. : 280

Sensitivity : 0.05 (full scale deflection corrosponds to an absorbance of 0.05)

ml/min: 0.35

cm/ml : 0.5

note : Pharmacia REC 482 charts have been reduced 2.4 X . Each division along the X axis corrosponds to 2 ml eluant

Figure 14 subheadings:

a: 8-S_6cont
b: 8-S_6-PPP (small 2% peak in 500-1000 kd range - see arrow)
c: 8-S_6-E

For each: column eluant collected with glass wool filtration; 8-Sup.6-PPP : 500 - 1000 contained bovine k.l.p. as identified by immunoblotting. No peaks were discernable in this region for any of the solutions.

Figure 14:

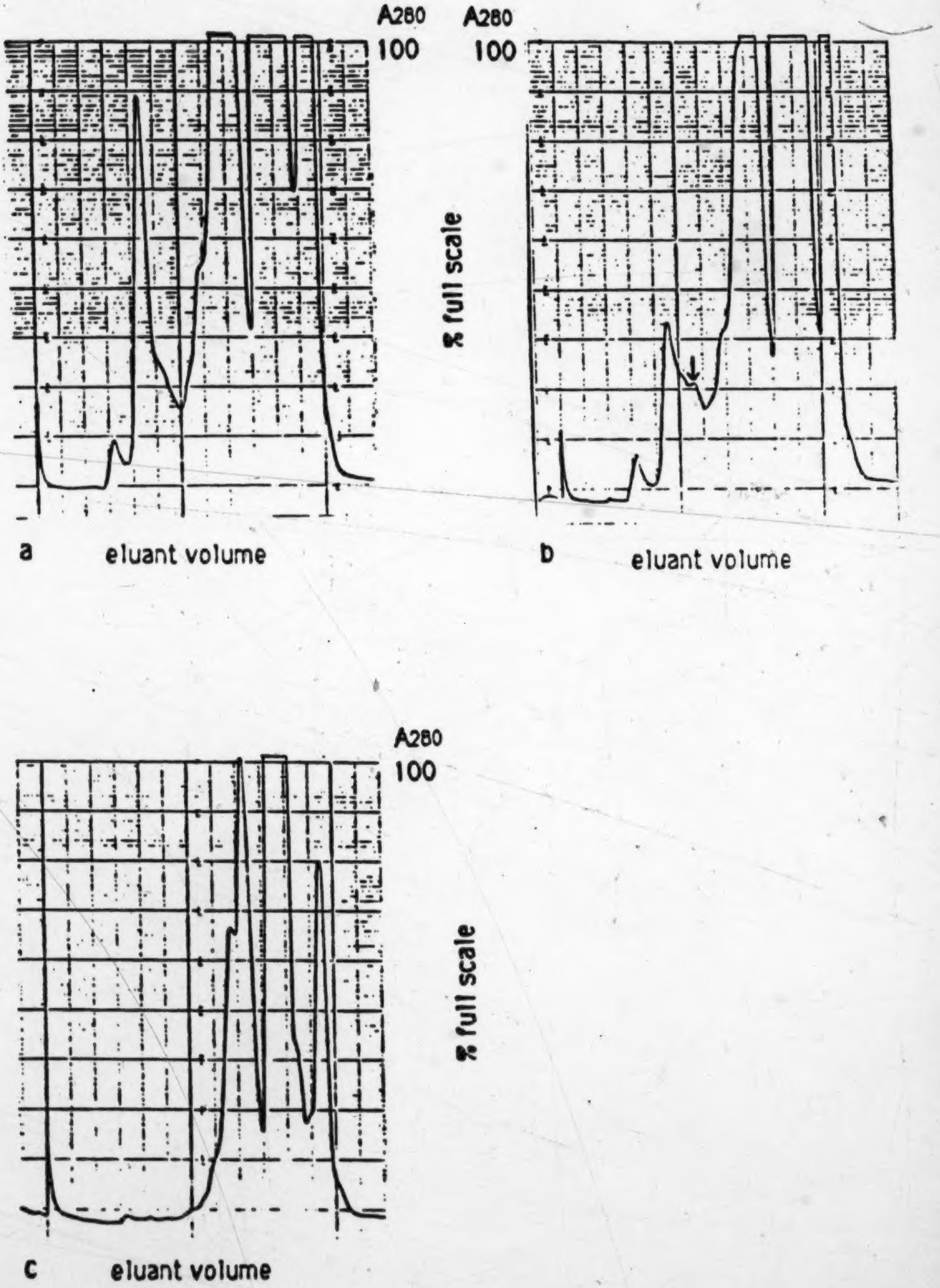

A280
100
A280
100
% full scale
a
eluant volume
b
eluant volume
A280
100
% full scale
c
eluant volume

PLATE XVIII: Run #8

Immunoblotting of Bovine Brain S_6 and Superose-6 Purified Bovine K.L.P.

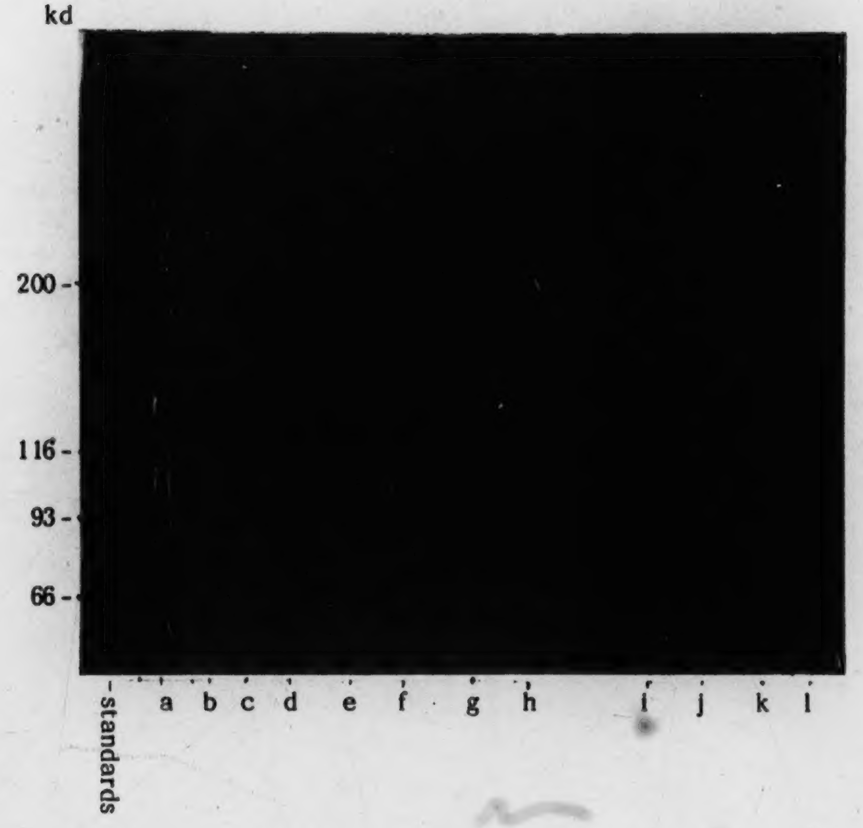
Standard procedure

normal rabbit serum (n.r.s.) control used

lane a : 8-Sup.6-E : >1,000
lane b : 8-Sup.6-E : 500-1,000
lane c : 8-Sup.6-PPP : >1,000
lane d : 8-Sup.6-PPP : 500-1,000 (contained bovine k.l.p.)
lane e : 8-S_6-PPP (contained bovine k.l.p.)
lane f : 8-S_6-E (contained bovine k.l.p.)
lane g : 8-S_6cont
lane h : 8-S_3
lane i : 8-S_6-PPP (n.r.s. control)
lane j : 8-S_6-E (n.r.s. control)
lane k : 8-S_6cont (n.r.s. control)
lane l : 8-S_3 (n.r.s. control)

It is interesting to note that in this run, n.r.s. identified a high molecular weight protein in 8-S_6-PPP, 8-S_6-E, 8-S_6cont, and 8-S_3. Random cross reactivity is proposed as an explanation.

Plate XVIII


kd
200 -
116 -
93 -
66 -
standards
a
b
c
d
e
f
g
h
i
j
k
l

IV. DISCUSSION

A. MICROTUBULES

Microtubule purification was originally performed using the reversible assembly method of Borisy et al., (1975). Microtubules in crude brain homogenate will assemble at 37°C and disassemble at 0°C; these characteristics were used to create a repeated assembly/disassembly cycle which purified tubulin. High speed centrifugations at 0°C removed cellular debris from the homogenate; subsequent centrifugations at 37°C caused polymerized microtubules to pellet. Resuspension of the microtubule pellet at 0°C and a repetition of the two centrifugation steps cleaned the microtubules.

The use of taxol in microtubule purification allowed higher purity and higher yields than those attainable with the reversible assembly method. Taxol is an antimitotic agent derived from the Western yew plant (Wani et al., 1971) that dramatically stimulates the polymerization of mammalian cytoplasmic microtubules (Schiff et al., 1979). Vallee (1982) developed a taxol-dependent procedure for the rapid isolation of microtubules and microtubule-associated proteins (MAPs) (see Figure 4, page 39). Vale et al., (1985c) used the microtubule purification procedure of Vallee (1982) to obtain clean microtubules for use in the microtubule affinity purification of kinesin. We also used the procedure of Vallee (1982), since it was superior to any available microtubule purification technique. Our taxol was a gift from Matthew Suffness of the National Cancer Institute.

Our results show that we were successful in purifying clean, MAP-free microtubules from bovine brain.

B. ISOELECTRIC FOCUSING

We initially chose isoelectric focusing for characterizing our protein-containing solutions. Isoelectric focusing is perhaps the most ingenious and effective electrophoretic method available for separating proteins. The mixture of proteins is subjected to an electric field in which a pH gradient has first been generated. Each protein migrates toward and is sharply focused at that portion of the pH gradient where the pH is equal to its isoelectric point. Very high resolution is available with this technique.

As well as the high resolution obtainable with IEF, there were other advantages which made the method attractive. Electrophoretic separation and gel staining is less time-consuming with IEF than with polyacrylamide gel electrophoresis. Also, since agarose-IEF is a non-denaturing process, proteins would be isolated in their native form, which would allow further k.l.p. characterization. For example, tests for k.l.p. ATPase activity could have been performed directly on the gel (see Discussion, page 138).

However, IEF of k.l.p. failed to reveal differences between experimental and control S_6 (purified k.l.p. solutions). These same solutions, when run on SDS-PAGE (see Results, Run #1) showed a distinct band at 120 kd in experimental S_6 that was not present in control S_6. Since kinesin attaches to carboxylated latex beads, it seems probable that kinesin carries a native positive charge. Since we used ampholytes with a pH range of 3-10 and since no difference was seen between control and experimental S_6 solutions in this pH range, we propose that the isoelectric point of bovine k.l.p. is above pH = 10. This would give bovine k.l.p. a positive charge at neutral pH. Unfortunately, we were unable to obtain ampholytes that would induce a pH above 10, so an exact value for the pI of bovine k.l.p. was not obtainable.

C. SDS-PAGE

We also used sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze our protein containing solutions. This was a very successful technique; we were able to show the distinct presence of an extra 120 kd subunit protein in experimental S_6. Sometimes this band appeared as a singlet, and sometimes as a doublet. This could have been due to subtle variations in gel casting technique, even though the gels involved were cast and run using a standardized procedure. Coomassie blue staining proved to be an adequate protein detection technique. Silver staining was also tried but found to be unnecessary, and sometimes inconsistent in results obtained.

D. ION EXCHANGE COLUMNS

The techniques used with ion exchange columns were not detailed in the Materials and Methods section, since no presentable results were obtained with this method. Briefly, anion exchange columns contain positively charged elements which are bound to the column's matrix. Sample solutions are injected onto the top of the column. The positively-charged elements interact with negatively charged proteins present in the sample solution. More negatively-charged proteins bind more strongly to the column's matrix than less negatively charged proteins, wheras positively charged proteins do not bind at all and are eluted with the void volume of the column. Bound proteins are selectively released and eluted by a salt gradient in the column buffer.

Cation exchange columns contain negatively charged elements bound to the column's matrix that bind positively charged proteins. Selective salt elution separates the bound proteins.

The PAGE analysis had shown proof of the existence of an extra protein in the experimental S_6 solutions, and the IEF gave some clue as to its pI. We attempted to use an anion exchange column (Pharmacia : Mono Q) to detect bovine k.l.p.. A highly basic buffer (pH = 10) was used. This gave most of the proteins in the S_6 a negative charge, causing them to bind to the positively charged matrix of the column. IEF results and knowledge of the protein's glass-binding behavior indicated that bovine k.l.p. would probably still carry a positive charge at pH = 10. It would thus not bind to the column and would elute with the void volume. No extra peaks were seen in the void volume for experimental vs control S_6. As described in the discussion for gel filtration, the most probable reason for the lack of an extra peak in k.l.p. containing solutions was loss due to adsorption; k.l.p. probably binds to the glass walls of the column. It is also possible that the high pH used with the anion exchange column caused denaturation of the k.l.p., preventing it from eluting as a single easily distinguishable peak. Also, the low concentration of k.l.p. in the S_6 solution (see Discussion, page 141) probably played a role in the absence of a detectable k.l.p. peak in experimental S_6.

We next tried a cation exchange column (Pharmacia - Mono S), using a buffer with a neutral pH to avoid denaturing problems. Again, no extra peaks were seen experimental vs control S_6. Peak identification was complicated by the fact that we did not know at what salt concentration bovine k.l.p. should be eluting from the column, since we did not know the pI of this protein. Again, possible column losses due to adsorption and low S_6 k.l.p. concentration could have played roles.

E. GEL FILTRATION

Next we switched to a gel filtration column, since we had a reported molecular weight for bovine k.l.p. (600 kd - Vale et al., 1985c) and could therefore approximate the elution volume for the protein. As described in Materials and Methods (page 51), we initially used the column buffer common to Piazza and Stearns (1986) and Vale et al., (1985c) (0.1 M KCl, 0.05 M Tris, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA pH = 7.6). This buffer was used both with and without ATP and EDTA. With each buffer tried, an 80-90 % column loss of k.l.p. was noticed. The only change noted was the dramatic effect of ATP on the baseline readout from the spectrophotometer. The inclusion of SDS in the column buffer, which was intended to swamp all non-specific binding sites on the column and neutralize all charges on the k.l.p. proteins, did not prevent column losses of k.l.p..

We did not solve the problem of column losses. It is interesting to analyze the results of other investigators on this topic. For instance, Vale et al., (1985c) used a Bio-Gel A5m column to purify bovine k.l.p.. If this gel was used in a glass walled column, a k.l.p. loss during gel filtration would be expected. Originally a 750 µl sample, containing (according to their figures) 750 to 1500 µg of bovine k.l.p. was injected onto the column. From pictures of their SDS-PAGE gels, which included gel scanning performed on one lane, I estimate that 150 µg of k.l.p. was present in the column eluant from this 750-1500 µg sample solution. This is an 80-90% loss during gel filtration, which corresponds to our results. They do not discuss this column loss. Apparently the large amounts of k.l.p. present in the S_6 allowed motility tests and SDS-PAGE identification of post-column bovine k.l.p., despite column losses. The reason for the large discrepancy in the concentration of bovine k.l.p. in our S_6 as compared to their S_6 is discussed elsewhere (see Discussion, page 141).

F. AFTER-COLUMN CONCENTRATION

We could not directly identify k.l.p. in eluant from the Superose 6 column that was run directly on SDS-PAGE. Again, this was probably due to the low amounts of k.l.p. in our S_6 solutions and to column losses. We therefore experimented with various ways of concentrating proteins in column eluant solutions. Centricon microconcentrators, treated or not with BSA, (see Materials and Methods, page 62) were unsuccessful; no 120 kd subunits of bovine k.l.p. were identified after concentration of gel eluant. According to Bruce Crise, a technician in Bruce Schnapp's lab (the Marine Biological Laboratory, Woods Hole, MA), during Centricon 10 concentrations of 5-8 ml of 1 mg/ml squid kinesin, up to 90% losses were experienced. This concentrator loss was not reported by Vale et al., (1985c) who used BSA treated Centricon 30 units to equilibrate k.l.p. containing fractions from a hydroxyapatite column with motility buffer.

We also used freeze drying to concentrate our proteins from column eluant. This procedure did not produce detectable amounts of k.l.p..

Since bovine k.l.p. was known to adsorb to glass, we decided to try filtering column eluant through glass wool as a method of concentrating the protein. When the bound proteins were released by boiling in Treatment Buffer (see Materials and Methods, page 65) and run on SDS-PAGE, bovine k.l.p. was found in the fraction corresponding to 500-1000 kd. This technique was successful, but yielded only denatured bovine k.l.p.. This was adequate for

PAGE and immunoblot analysis, but different methods would need to be developed if k.l.p. generated microtubule or bead motility were an objective.

Glass wool filtration was used as the standard procedure for identifying k.l.p. in Superose 6 gel filtration eluant; this technique could have conceivably been used to identify k.l.p. in ion exchange column eluant as well. Ion exchange columns are capable of higher sample volumes than gel filtration columns. Had we had more time, more powerful FPLC methods could have been developed. For example, high volume ion-exchange separations of high-speed homogenate followed by gel filtration of the ion exchange eluant fractions that contained k.l.p. could conceivably have bypassed microtubule-affinity purification entirely.

G. NON-DENATURING DISCONTINUOUS GELS

Ekstrom and Kanje (1986) performed ATPase assays of proteins directly upon polyacrylamide gels. We thought this would be an ideal way to correlate ATPase activity with bovine k.l.p.. IEF separation of experimental and control S_6 did not give a band for bovine k.l.p. (see Discussion, page 133). We therefore developed a non-denaturing PAGE technique to allow the electrophoresis of non-denatured bovine k.l.p. (see Materials and Methods, page 71). It became apparent as studies progressed that a non-denatured gel ATPase assay might not work with bovine k.l.p., since studies by Kuznetzov and Gefland (1986) and others (see Introduction, page 21) indicated that the presence of microtubules or high calcium levels were necessary for ATPase activity to occur. High calcium levels could have been produced in the gel, but difficulties encountered in the casting and running of gels limited progress. Moreover, in non-denaturing PAGE, the proteins did not load well onto the gels, and only very diffuse protein bands were produced. This approach was therefore abandoned.

H. IMMUNOLOGICAL IDENTIFICATION

At this point, we had a suspected bovine k.l.p. that had been characterized by Superose 6 gel filtration and SDS-PAGE. We also had a suspected canine k.l.p. that had been characterized by SDS-PAGE. We considered three possible ways of positively identifying these proteins as a true k.l.p.

The most obvious method was to use purified k.l.p. to promote the movement of latex spheres on microtubules or microtubules on glass. As described earlier, Vale et al., (1985c) found that in order for bovine k.l.p. to promote microtubule movement, it was necessary to purify bovine brain S_6 via gel filtration. We tried using both S_6 and S_6 gel filtration column eluant to promote motility, but neither worked. The possible reason for this may be the low concentration of bovine k.l.p. in our gel eluant. Vale et al., (1985c) found that column-purified bovine k.l.p. could induce microtubule movement at concentrations as low as 5 µg/ml. Based upon the glass-wool retrieval method, we estimated the concentration of bovine k.l.p. in our gel eluant to be 0.3-0.5 µg/ml, an order of magnitude below the minimum concentration established by Vale et al., (1985c). Again, this low concentration was due largely to column losses and low S_6 k.l.p. concentration.

Another way to positively identify our k.l.p. would have been to measure ATPase activity. Kuznetsov and Gefland (1986) used only ATPase activity and molecular weight characteristics to positively identify bovine brain k.l.p.. However, as mentioned earlier (see page 138) performing ATPase tests on IEF or nondenaturing discontinuous gels was not possible due to the dependence of bovine k.l.p.'s ATPase activity on the presence of microtubules or high calcium levels. Performing ATPase tests on k.l.p.-containing solutions was considered. Kuznetsov and Gefland (1986) showed ATPase activity in a solution containing 1.0 mg/ml microtubules, 9.4 µg/ml bovine k.l.p. and 15 µM taxol. However, since ATPase activity of kinesin and k.l.p.'s is a controversial area (see Introduction, page 21) pursuit of this evidence could have been time consuming.

We therefore opted for imunoblotting, which was our third alternative. Using this procedure, we were able to positively identify the 120 kd bovine k.l.p. subunit in S_6 fractions from EDTA, AMP-PNP and PPP runs, in S_2 (high speed homogenate) solution, and in glass-filtration collected Superose 6 column eluant from an 8-S_6-PPP sample (see Results - Run #8). We also identified the 116 kd subunit from squid kinesin. The fact that Rabbit anti-Squid kinesin cross reacts with bovine k.l.p. suggests that epitopes containing kinesin's structural information have been carefully conserved throughout evolution - squid and cattle are phylogenically far apart, yet the neurological kinesin/k.l.p. from these two species are cross reactive.

I. PURIFICATION TECHNIQUES - ALTERNATIVES

1. S_6 K.L.P. Concentration

We were able to isolate a bovine k.l.p. as identified by PAGE, Superose 6 gel filtration and immunoblotting. However, the concentration of this protein in our final S_6 solution (average = 40 µg/ml) was an order of magnitude less than that reported by Vale et al., (1985c) for bovine k.l.p. (1-2 mg/ml). The reasons for our low yield were mysterious, since our basic procedure was identical to that outlined by Vale et al., (1985c), including such details as the type of homogenizer used.

We tried several alterations to the basic procedure in an attempt to increase k.l.p. yield. One of the most obvious variations was altering the ratio between S_1 and S_6. Vale et al., (1985c) do not precisely define the S_1:S_6 ratio used in bovine brain k.l.p. purifications, however it may be estimated to be between 20:1 and 70:1. We tried ratios from 20:1 to 60:1. No notable increase in S_6 k.l.p. concentration could be seen as the ratio was increased. This implied that a limiting factor was present, since an increasing S_1:S_6 ratio should cause an increased S_6 k.l.p. concentration.

One possible limiting factor was that clean microtubules were always added to the S_3 in a 100 µg/ml proportion. The S_3 bovine k.l.p. may have been present in large quantities, but a certain concentration of k.l.p, could have saturated the k.l.p. binding sites on these microtubules. This would result in a constant amount being released into the final S_6 solution, irrelevant of the amount of k.l.p. in th crude homogenate. However, Vale et al., (1985c) also used 100µg/ml clean microtubules; unless our microtubules were different than theirs, there is no apparent reason why our k.l.p. yield should have been lower. Our analysis of our bovine brain microtubules showed that they were clean, containing no MAP contaminants.

Also, the results of our purification runs performed with endogenous microtubules implied that microtubule saturation was not a limiting factor for S_6 k.l.p. concentration. The use of endogenous instead of clean microtubules during k.l.p. purification meant that 10 X more microtubules, or about 1000 µg/ml, were available in the S_3 for k.l.p. binding (tubulin comprises 0.1% of the brain's mass - see Materials and Methods, page 40). Since endogenous microtubules are associated with MAPs and clean microtubules are not, no direct comparison between the k.l.p. concentrations produced by the two methods can be made. However, S_6 k.l.p. concentration remained at about 10-40 µg/ml when endogenous microtubules were used in the purification. This led to two conclusions. First, endogenous microtubules may be used in the purification of bovine k.l.p.. This is important, since it shows that k.l.p. can bind to microtubules that are associated with MAPs. It is likely that *in vitro* axonal microtubules also interact with both MAPs and k.l.p.. The second conclusion is that either endogenous microtubules are only 10% as effective as clean microtubules in binding k.l.p., or the amount of microtubules present in the S_3 did not limit the k.l.p. yield.

It is possible that the laboratory materials we used caused a loss of k.l.p. during the purification. K.l.p. binds tightly to glass and carboxylated latex beads, and may have adsorbed to the Beckman Polyallomer (3226814) high speed centrifuge tubes, the polypropylene Servall SS-34 centrifuge tubes, the Fisher 1.5 ml microcentrifuge tubes or the Fisher 1-100 µl Eppendorf micropipette tips. Not many material options were available for these pieces of labware, and since no mention of special materials was made by Vale et al., (1985c), we assumed these common materials were adequate. Perhaps this deserves further investigation.

In conclusion, adequate answers to the problem of low k.l.p. yield were not found. It is tempting to suggest that the estimates of bovine k.l.p. concentration quoted by Vale et al., (1985c) were inaccurate. An examination of their SDS-PAGE results does not provide unquestionable proof for 1-2 mg/ml S_6 bovine k.l.p. concentrations. However, their success in generating microtubule motility using gel filtration eluant suggests that either their column losses were lower than ours or their S_6 k.l.p. concentrations were higher than ours.

2. Binding Agent Changes

Other alterations made to the basic procedure were more successful. During discussions on kinesin's possible mechanism, it was pointed out that as an ATPase, kinesin was probably Mg^{++} dependent. Examining the structure of known ATPases suggested that chelating Mg^{++} would likely halt the ATPase cycle, and possibly bind k.l.p. to microtubules. Ethylenediamine tetraacetate (EDTA) was chosen as a chelating agent, and purifications were performed substitutuing EDTA for AMP-PNP. This purification technique was successful.

The question of how Mg^{++} chelation causes k.l.p. binding has two possible answers, according to the theoretical ATPase cycle for kinesin proposed by Hill (1986) (see Figure 3, page 32). Mg^{++} chelation could prevent ATP from assuming the correct tertiary configuration necessary for interaction with kinesin's ATPase site. This would create a situation analogous to ATP depletion, which has been suggested as being a condition which causes strong k.l.p. binding (see Introduction, page 12).

A second possible interpretation would be that the lack of Mg^{++} does not prevent the ATP from entering the ATPase site, but does prevent the k.l.p. from hydrolyzing the ATP. In this interpretation, Mg^{++} chelation causes ATP to become a non-hydrolyzable analogue of MgATP, similar to AMP-PNP. Evidence against this interpretation comes from recent work by Vale et al., (1986b) in which the effect of various buffers on kinesin-induced microtubule movement along glass was studied. Movement was stopped by incubation in 5 mM AMP-PNP, 0 ATP or 0 Mg solutions. However, recovery of movement once MgATP was introduced was much faster for the 0 ATP and 0 Mg-stalled microtubules than for the AMP-PNP-stalled microtubules. This implies that the binding condition created by the 0 ATP and 0 Mg buffers are analagous to each other and different than the binding condition created by AMP-PNP. It is also conceivable that if 0 Mg was causing ATP to act as a non-hydrolyzable analogue of MgATP, the reintroduction of Mg^{++} into the medium allowed an ATP molecule already locked into the ATPase site to be rapidly released; however, this seems less likely.

Large amounts (40 mM) of EDTA were necessary for inducing maximum k.l.p. binding. One mM $MgSO_4$ was present in the homogenization buffer, but this high EDTA concentration (40 mM) is surprising. High levels of EDTA (10 mM) are also necessary to block anterograde transport of particles in *Xenopus* axons (Smith, 1987, personal communication). Intracellular fluid contains 30-60 mEq/L Mg^{++} (Guyton, 1971), which is quite high; this may expain the high requirement for EDTA. Any further comment upon this topic must await clarification of the mechanism of k.l.p.'s action.

Another binding agent we used in purifications was tripolyphosphate (PPP). The originators of this technique (Kuznetsov and Gefland, 1986) did not examine the possible mechanism for this binding agent's action. It is likely that it works on the same Mg^{++} chelating principle proposed for EDTA, since at pH = 7, the log K_1 for the Mg^{++}-association reaction for EDTA (5.4) and PPP (3.7) are both large (Dawson et al., 1969; pages 426 and 430). The molecule is quite small, and therefore probably not suitable for acting as a non-hydrolyzable analogue of ATP.

Since the concentration of ATP is a critical factor to these discussions, the careful monitoring of ATP concentration would possibly shed light upon the mechanism of k.l.p. binding. Since our homogenization buffers contained no nucleotides, any ATP present in the crude homogenate would be endogenous in origin. It would have been interesting to perform k.l.p. purifications using different ATP concentrations. We would predict that isolation procedures dependent upon Mg^{++} would be relatively insensitive to ATP levels in the crude homogenate, since a rigor-like state has already been induced. The only difference would be in the background levels of binding in control solutions; 0 ATP has been described as a state causing strong k.l.p. binding to microtubules (Vale et al., 1986b), and very low concentrations of ATP in the crude homogenate would likely lead to the presence of k.l.p. in control S_6.

Isolation procedures dependent upon the presence of a competitively-binding non-hydrolyzable analogue of ATP, on the other hand, would probably be affected by ATP levels in the crude homogenate. Amos (1987) used hexokinase + glucose to reduce the levels of ATP present in crude pig brain homogenate. This reduced the AMP-PNP requirement for the microtubule-affinity purification of pig brain k.l.p. to 0.5 mM [Vale et al., (1985c) originally used 5 mM AMP-PNP to purify bovine k.l.p. from non-ATP depleted crude homogenate]. This could be a way to determine the binding mechanism for PPP; a series of purifications with a graded amount of ATP in the crude homogenate would show whether PPP binding was competitive with ATP or whether PPP was causing Mg^{++} depletion.

3. Alternative Tissues

Tissues other than dog and cow brain were also used in k.l.p. purifications. Rabbit kidney and liver, cow kidney and liver, pig brain and mandibular gland, and dog spinal cord k.l.p. purifications were also attempted. Secretory organ tissue k.l.p. purifications (kidney, liver and mandibular gland) were performed in the hope of showing the presence of k.l.p. in tissues whose functions were not neurological; this would provide evidence of a more general role for k.l.p.. Purifications of k.l.p. from rabbit kidney and liver, cow kidney and liver, and pig mandibular gland were unsuccessful in our lab. However, microtubule affinity purification has recently been used by other investigators to purify k.l.p. from rabbit kidney and liver (Vale et al., 1986a). The presence of k.l.p.'s in a variety of other tissues, including sea urchin eggs (Scholey et al., 1985) and DU 145 human tumor cells (Piazza and Stearns, 1986) does suggest that k.l.p.'s have a generalized function within cells (see Figure 2, page 18).

We were able to purify a suspected k.l.p. from dog brain, dog spinal cord and pig brain. One feature that seemed to dictate the success of k.l.p. purification for all tissues was the age of the animal from which the tissue had been taken. Old dogs (7-8 years) and old hiefers (5-6 years) had lower levels of brain k.l.p. than young dogs (1-2 years) and young heifers (1-1 1/2 years). Young pigs (1 year) had higher levels of brain k.l.p. than very young pigs (3-4 months) (results not shown). It is possible that the state of neurological development or degeneration is a determining factor in the level of k.l.p. present in the brain; further studies on this topic are highly recommended.

J. CONCLUSION: SIMPLIFIED K.L.P. PURIFICATION TECHNIQUE

The original microtubule-affinity technique for the isolation of kinesin from squid axoplasm and k.l.p. from bovine brain (Vale et al., 1985c) has been used by other investigators for the isolation of k.l.p.'s from a variety of tissues (see Figure 2, page 18). We have used this basic technique for the isolation of bovine k.l.p. and have examined a number of alternative strategies for simplifying and improving the procedure. The following conclusions may be made:

1. Homogenization buffer: The homogenization buffer can be a simple buffered medium if all the purification steps are to be completed in a single day. If freezing and storage of crude or high speed homogenates are to be carried out, we recommend the inclusion of protease inhibitors in the buffer. Our recipe and the recipes of other investigators appear in the homogenization buffer survey (see Materials and Methods, page 49).

2. Microtubules: The use of endogenous microtubules (see Figure 6, page 53) simplifies the isolation procedure and does not cause noticable contamination of the S_6. No effect on the S_6 concentration of k.l.p. is apparent.

3. Washes: The standard usage of 1 wash is recommended, since eliminating this wash causes an increase in the amount of contaminating proteins in the S_6. An additional wash does not give a worthwhile improvement in the cleanliness of the S_6.

4. Binding agents: EDTA and PPP are recommended as ideal binding agents, since the cost of these chemicals is far below the cost of AMP-PNP. The purification of k.l.p. from 5 ml of S_3 using 5 mM AMP-PNP (or 12.5 mg) uses $30.00 worth of the nucleotide to isolate about 10 µg of k.l.p. (our results). This corresponds to $3000/mg or $3,000,000/gram of bovine k.l.p.. In contrast, using EDTA costs approximately $0.37/mg or $3.70/gram of bovine k.l.p., and the cost of using PPP is even lower.

5. S_1:S_6 ratios: Changing this ratio had no noticable effect on final S_6 k.l.p. concentration.

V. REFERENCES

Adams, R.J. (1982), Organelle movement in axons depends on ATP,*Nature* **297:** 327-329.

Allen, R.D., Allen, N.S., Travis, J.L. (1981), Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: a new method of analyzing microtubule-related motility in the reticulo podial network of*Allogromia laticollaris, Cell Motil.* **1:** 291-302.

Allen, R.D., Metuzals, J., Tasaki, I., Brady, S.T., and Gilbert, S.P. (1982), Fast axonal transport in squid giant axon, *Science* **218:**1127-1129.

Ambron, R.T., Goldman, J.E., and Schwartz, J. (1974), Axonal transport of newly synthesized glycoproteins in a single identified neuron of*Aplysia californica, J. Cell Biol.* **61:** 665-675.

Amos, L.A. (1987), Kinesin from pig brain studied by electron microscopy, *J.Cell Sci.* **87:** 105-111.

Black, M.M., and Lasek, R.J. (1979), Axonal transport of actin: slow component b is the principal source of actin for the axon, *Brain Res.* **171:** 401-413.

Black, M.M., and Lasek, R.J. (1980), Slow components of axonal transport: two cytoskeletal networks, *J. Cell Biol.* **86:** 616-623.

Borisy, G.G., Markum, J.M., Olmsted, J.B., Murphy, D.B., and Johnson, K.A. (1975), Purification of tubulin and associated high molecular weight proteins porcine brain and characterization of microtubule assembly *in vitro, Ann. N.Y. Acad. Sci. USA.* **72:** 3570-3574.

Brady, S.T., and Lasek, R.J. (1981), Nerve-specific enolase and creatine phosphokinase in axonal transport: soluble proteins and the axoplasmic matrix, *Cell* **23:** 515-523.

Brady, S.T., Lasek, R.J., and Allen, R.D. (1982), Fast axonal transport in extruded axoplasm from squid giant axon, *Science* **4577:** 1129-1131.

Brady, S.T. (1985), A novel brain ATPase with properties expected for the fast axonal transport motor, *Nature* **317:** 73-75.

Brady, S.T., Lasek, R.J., and Allen, R.D. (1985), Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms, *Cell Motil.* **5:** 81-101.

Collins, C.A., and Vallee, R.B. (1986), A microtubule-activated ATPase from sea urchin eggs, distinct from cytoplasmic dynein and kinesin, *Proc. Natl. Acad. Sci. USA,* **83:** 4799-4803.

Cooper, P.D., and Smith, R.S. (1974), The movement of optically detectable organelles in myelinated axons of *Xenopus laevis*, *J. Physiol. (London)* **242:** 77-97.

Dahlstrom, A. (1965), Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression, *J. Anat.* **4:** 677-689.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H., Jones, K.M., eds. (1969), *Data for Biochemical Research,* Second Edition, Oxford University Press, London.

Droz, B., and Leblond, C.P. (1962), Migration of proteins along the axons of the sciatic nerve, *Science* **137:** 1047-1048.

Droz, B., and Leblond, C.P. (1963), Axonal migration of proteins in the central nervous system and peripheral nerves as shown by radioautography, *J. Comp. Neurol.* **121:** 325-346.

Ekström, P., Kanje, M. (1986), Properties of erythro-9-(3-(2-hydroxynonyl)) adenine (EHNA)-binding proteins from rabbit brain, Abstract from Axonal Transport; A Satellite Symposium of the XXX International Congress International Union of Physiological Sciences, University of Calgary, July 9-12, 1986.

Fontana, F. (1778), *Treatise on the Venom of the Viper; on American Poisons; and on the Cherry Laurel and Some Other Vegetable Poisons to Which Are Annexed Observations on the Primitive Structure of the Animal Body; Different Experiments on the Reproduction of the Nerves and a Description of the Nerves; and a New Description of a New Canal of the Eye*, two volumes, translated from the French by J. Skinner, Murray, London.

Forman, D.S., Brown, K.J., and Livengood, D.R. (1983), Fast axonal transport in permeabilized lobster giant axons is inhibited by vanadate, *J. Neurosci.* **3:**1279-1288.

Forman, D.S., New approaches to the study of the mechanism of fast axonal transport, *TINS,* April, 1984,112-116.

Forman, D.S., Padjen, A.L., and Siggins, G.R. (1977), Axonal transport of organelles visualized by light microscopy: Cinemicrographic and computer analysis, *Brain Res.* **136:**197-213.

Gilbert, S.P., Allen, R.D., and Sloboda, R.D. (1985), Translocation of vesicles from squid axoplasm on flagellar microtubules, *Nature* **315:** 245-248.

Gilbert, S.P., and Sloboda, R.D. (1984), Bidirectional transport of fluorescently labeled vesicles introduced into extruded axoplasm of squid *Loligo pealei*, *J. Cell Biol.* **99:** 445-452.

Gilbert, S.P., and Sloboda, R.D. (1986), Identification of a MAP 2-like ATP binding protein associated with axoplasmic vesicles that translocate on isolated microtubules, Abstract # 2060, *J. Cell Biol.*, **103.**

Goldman, J.E., Kim, K.S., and Schwartz, J.H. (1976), Axonal transport of (^{3}H) serotonin in an identified neuron of *Aplysia californica* , *J. Cell Biol.* **70:** 304-318.

Grafstein,B. (1967), Transport of protein by goldfish optic nerve fibers, *Science* **157:** 196-198.

Grafstein, B., and Forman, D.S. (1980), Intracellular transport in neurons, *Physiol Rev.* **60:**1167-1283.

Griffin, J.W., Fahnestock, K.E., Price, D.L., and Hoffman, P.N. (1983), Microtubule-neurofilament segregation produced by beta, beta'-iminodipropionitrile: evidence for the association of fast axonal transport with microtubules, *J. Neurosci* **3:** 557-566.

Gross, G.W. (1975), The microstream concept of axoplasmic and dendritic transport, *Adv. Neurol.* **12:** 283-296.

Harrison, R.G. (1910), The outgrowth of the nerve fiber as a mode of protoplasmic movement, *J. Exp. Zool.* **9:** 787-846.

Hayden, J.H., Allen, R.D., and Goldman, R.D. (1983), Cytoplasmic transport in keratocytes: direct visualization of particle translocation along microtubules, *Cell Motil.* **3:** 1-19.

Heidemann, S.R., Landers, J.M., and Hamborg, M.A. (1981), Polarity orientation of axonal microtubules, *J. Cell Biol.* **91:** 661-665.

Hendry, I.A., Stockel, K., Thoenen, H., and Iverson, L.L. (1974), The retrograde axonal transport of nerve growth factor, *Brain Res.* **68:** 103-121.

Hill, T.L. (1986), Kinetic diagram and free energy diagram for kinesin in microtubule-related motility, *Proc. Natl. Acad. Sci. USA* **83:** 3326-3330.

Hirokawa, N. (1982), Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by the quick-freeze, deep-etching method, *J. Cell Biol.* **94:**129-142.

Hoefer Scientific Instruments (1986), *Hoefer Scientific Instruments* (Catalogue), San Francisco, CA.

Huxley, H.E. (1969), The mechanism of muscle contraction. Recent structural studies suggest a revealing model for cross-bridge action at variable filament spacing, *Science* **164:** 1356-1366.

Inoué, S. (1981), Video image processing greatly enhances contrast quality and speed in polarization-based microscopy, *J. Cell Biol.* **89:** 346-356.

Koonce, M.P., Schliwa, M. (1985), Bidirectional Organelle Transport Can Occur in Cell Processes That Contain Single Microtubules, *J. Cell Biol.* **100:** 322-326.

Kristensson, K., Olsson, Y. (1971), Retrograde axonal transport of protein, *Brain Res.* **29:** 363-365.

Kuznetsov, S.A., and Gefland, V.I. (1986), Bovine brain kinesin is a microtubule-activated ATPase, *Proc. Natl. Acad. Sci. USA* **83:** 8530-8534.

Lasek, R.[J.] (1968), Axoplasmic transport in cat dorsal root ganglion cells: As studied with 3H-L-leucine, *Brain Res.* **7:** 360-377.

Lasek, R.J., Axonal transport: a dynamic view of neuronal structures, *TINS* April, 1980, 87-91.

Lasek, R.J., and Brady, S.T. (1985), Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP, *Nature* **316:** 645-647.

Ledbetter, M.C. and Porter, K.R. (1963), A microtubule in plant fine structure, *J. Cell Biol.* **19:** 239-250.

Lehninger, A.L. (1975), *Biochemistry*, Second Edition, Worth Publishers, Inc., New York.

Levine, J., and Willard, M. (1981), Fodrin: axonally transported polypeptides associated with the internal periphery of many cells, *J. Cell Biol.* **90:** 631-642.

Lorenz, J., and Willard, M. (1978), Subcellular fractionation of intra-axonally transported polypeptides in the rabbit visual system, *PNAS* **75:** 505-509.

Lubinska,L. (1964), Axoplasmic streaming in regenerating and in normal nerve fibers, *Prog. Brain Res.* **13:** 1-71.

Lye, R.J., Porter, M.E., Scholey, J.M., and MacIntosh, J.R. (1986), Identification of a microtubule-based cytoplasmic motor in the nematode *C. elegans*, Abstract # 2062, *J. Cell Biol.* **103.**

McEwan, B.S., and Grafstein, B. (1968), Fast and slow components in axonal transport of protein, *J. Cell Biol.* **38:** 494-508.

Miller, R.H., and Lasek, R.J. (1985), Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm, *J. Cell Biol.* **101:** 2181-2193.

Ochs, S. (1971), Characteristics and a model for fast axoplasmic transport in nerve, *J. Neurobiol.* **2:** 331-345.

Ochs, S. (1972), Fast transport of materials in mammalian nerve fibers, *Science* 176: 252-260.

Ochs, S. (1975), A unitary concept of axoplasmic transport based on the transport filament hypothesis, pp. 189-194, in: *Third International Congress on Muscle Diseases*, Bradley, W.G., Gardner-Medwin, D., and Walton, J.N. (eds.), Excerpta Medica, Amsterdam.

Ochs, S. (1982), *Axoplasmic Transport and its Relation to Other Nerve Functions* , John Wiley & Sons, New York.

Olmstead, J.B., and Lyon, H.D. (1981), A microtubule associated protein specific to differentiated neuroblastoma cells, *J. Biol. Chem.* **256:** 3507-3511.

Penningroth, S.M., Rose, P.M., and Peterson, D.D. (1986), Activation of ATPase activity in the presence of kinesin and microtubules, Abstract #2071, *J. Cell Biol.,* **103.**

Peters, A., Palay, S.L., and Webster, H. deF. (1976), *The Fine Structures of the Nervous System: The Neurons and Supporting Cells*, W.B. Saunders Co., Philadelphia.

Pharmacia Fine Chemicals (1982), *Isoelectric Focusing : principles & methods* , Pharmacia Fine Chemicals AB, Box 175, S-751 04 Uppsala 1, Sweden.

Piazza, G.A., and Stearns, M.E. (1986), Characterization of purified kinesin from cultured human prostatic tumor cells, Abstract #2065, *J. Cell Biol.,* **103.**

Porter, M.E., Scholey, J.M., Stemple, D.L., Vigers, G.P.A., Vale, R.D., Scheetz, M.P., and McIntosh, J.R. (1986), Characterization of the microtubule movement produced by sea urchin egg kinesin, *J. Biol. Chem.* **262:** 2794-2802.

Purves, D., and Lichtman, J.W. (1978), Formation and maintenance of synaptic connections in autonomic ganglia, *Physiol. Rev.* **58:** 821-862.

Ramon y Cajal, S. (1894), La fine structure des centres nerveux, *Proc. Roy. Soc. B* **55:** 444-468.

Ramon y Cajal, S. (1909), *Histologie du système nerveux de l'homme et des vertébrés,* Vol. 1, translated by Azoulay, L.; republished by Consejo Superior de Investigaciones Cientificas, Instituto Ramon y Cajal, Madrid (1952).

Remak, R. (1838), *Observations anatomicae et microscopicae de systematis nervosi structura,* Dissertation, Reims, Berolini.

Saxton, W.M., Yang, J., Porter, M.E., Scholey, J.M., Goldstein, L.S.B., and McIntosh, J.R. (1986), Isolation and characterization of kinesin from *Drosophila*, Abstract #2063, *J. Cell Biol.*, **103.**

Schiff, P.B., Fant, J., and Horwitz, S.B. (1979), Promotion of microtubule assembly *in vitro* by Taxol, *Nature* (Lond.), **277**: 665-667.

Schiff, P.B., and Horwitz, S.B. (1980), Taxol stabilizes microtubules in mouse fibroblast cells, *Proc. Natl. Acad. Sci.USA* **77:** 1561-1565.

Schmitt, F.O. (1968), II. The molecular biology of neuronal fibrous proteins, *Neurosci. Res. Prog. Bull.* **6:** 119-144.

Schnapp, B.J., and Reese, T.S. (1982), Cytoplasmic structure in rapid-frozen axons, *J. Cell Biol.* **94:** 667-669.

Schnapp, B.J., and Reese, T.S., New developments in understanding rapid axonal transport, *TINS* April,1986.

Schnapp, B.J., Sheetz, M.P., Vale, R.D., and Reese, T.S. (1984), Transport filaments from squid axoplasm do not contain filamentous actin, *J. Cell Biol.* **99:** 351a.

Schnapp, B.J., and Stuart, A.E. (1983), Synaptic contacts between physiologically identified neurons in the visual system of the barnacle, *J. Neurosci.* **3:** 1100-1115.

Schnapp, B.J., Vale, R.D., Sheetz, M.P., and Reese, T. (1985), Single microtubules from squid axoplasm support bidirectional movement of organelles, *Cell* **40:** 455-462.

Schnapp, B.J., Vale, R.D., Sheetz, M.P., and Reese, T.S. (1986), Microtubules and the mechanism of directed organelle movement, *Ann NY Acad. Sci.* **466:** 909-918.

Scholey, J.M., Porter, M.E., Grissom, P.M., and McIntosh, J.R. (1985), Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle, *Nature* **318:** 483-486.

Scholey, J.M., Porter, M.E,, Neighbors, B.W., Grissom, P.M., McKenzie, K., Leslie, R., and McIntosh, J.R. (1986), Sea urchin egg kinesin, Abstract #2064, *J. Cell Biol.* **103.**

Schroer, T.A., and Sheetz, M.P. (1986), Identification of kinesin binding proteins in squid brain, Abstract #2074, *J. Cell Biol.* **103.**

Schwab, M., and Thoenen, H. (1977), Selective trans-synaptic migration of tetanus toxin after retrograde axonal transport in peripheral sympathetic nerves: A comparison with nerve growth factor, *Brain Res.* **122:** 459-474.

Schwab, M.E., Suda,K., and Thoenen, H. (1979), Selective retrograde transynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport, *J. Cell Biol.* **82:** 798-810.

Schwab, M.E., and Thoenen, H. (1983), Mechanism of uptake and retrograde axonal transport of nonadrenaline in sympathetic neurons in culture: Reserpine-resistant large dense-core vesicles as transport vehicles, *J. Cell Biol.* **96:** 1538-1547.

Scott, F.H. (1905), On the metabolism and action of nerve cells, *Brain* **28:** 506-526.

Sheetz, M.P., Schnapp, B.J., Vale, R. and Reese, T.S. (1984), Organelle transport in squid axoplasm: inhibition by drugs that collapse proton gradients, *J. Cell. Biol.* **99(4, Pt.2):** 118a

Sheetz, M.P., and Spudich, J.A. (1983), Movement of myosin-coated fluorescent beads on actin cables in vitro, *Nature* **303:** 31-35.

Singer, S.J., and Nicolson, G.L. (1972), The fluid mosaic model of the structure of cell membranes, *Science* **175:** 720-731.

Smith, R.S. (1980), The short term accumulation of axonally transported organelles in the region of localized lesions of single myelinated axons, *J. Neurocytol* **9:** 39-65.

Smith, R.S. (1986), Evidence that individual vesicles switch driving mechanisms during reversal of rapid axonal transport, Abstract #2075, *J. Cell Biol.*, **103.**

Smith, R. S. (1987), Control of the direction of rapid axonal transport in the vertebrates, in: *Axonal Transport,* Smith, R.S., and Bisby, M.A. (eds.), Alan R. Liss, Inc., New York.

Spencer, P.S. (1972), Reappraisal of the model for "bulk axoplasmic flow," *Nature (New Biol.)* **240:** 283-285.

Stoeckel, K., Paravicini, U., and Thoenen, H. (1974), Specificity of the retrograde axonal transport of nerve growth factor, *Brain Res.* **76:** 413-421.

Stoeckel, K., Schwab, M., and Thoenen, H. (1975), Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study, *Brain Res.* **89:** 1-14.

Thomas, J.M. and Hodes, M.E. (1981), A new discontinuous buffer system for the electrophoresis of cationic proteins at near-neutral pH. *Anal Biochem.* **118(1):** 194-196.

Tilney, L.F., Bryan, J., Bush, D.J., Fujiwara, K., Moosekar, M.S., Murphy, D.B., and Snyder, D.H. (1973), Microtubules: Evidence for 13 protofilaments, *J. Cell Biol.* **59:** 267-275.

Tsukita, S., and Ishikawa, H. (1980), The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles, *J. Cell Biol.* **84:** 513-530.

Vale, R.D., Schnapp, B.J., Reese, T.S., and Sheetz, M.P. (1985a), Movement of organelles along filaments dissociated from the axoplasm of the squid giant axon, *Cell* **40:** 449-454.

Vale, R.D., Schnapp, B.J., Reese, T.S., and Sheetz, M.P. (1985b), Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell* **40:** 559-569.

Vale, R.D., Reese, T.S., and Sheetz, M.P. (1985c), Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility, *Cell* **42:** 39-50.

Vale, R.D., Schnapp, B.J., Mitchison, T., Steuer, E., Reese, T.S., and Sheetz, M.P. (1985d), Different axoplasmic proteins generate movement in opposite directions along microtubules *in vitro*, *Cell* **43:** 623-632.

Vale, R.D., and Reese, T.S. (1986), Proteolytic fragments of kinesin with microtubules binding activity, Abstract #2070, *J. Cell Biol.* **103.**

Vale, R.D., Scholey, J.M., and Sheetz, M.P. (1986), Kinesin: possible biological roles for a new microtubule motor, *TIBS*, **11:** 464-468.

Vallee, R.B. (1982), A taxol-dependant procedure for the isolation of microtubules and microtubule-associated proteins (MAPS), *J. Cell Biol.* **92:** 435-442.

van Leeuwenhoek, A. (1717), Letter to Abraham van Bleiswyk (March 2, 1717) printed in "Epistolae physiologicale super compluribus naturae arcanis" (Beman, Delft, 1719), Epistola XXXII, pp. 310-327, and in part translated pp. 32-35, in: *The Human Brain and Spinal Cord*, Clarke, E., and O'Malley, C.D. (eds.), University of California Press, Berkley, 1968.

Wagner, M.C., Bloom, G.S., and Brady, S.T. (1986), Purification and physical characterization of an apparent motor for fast axonal transport, Abstract #2067, *J. Cell Biol.*, **103.**

Waller, A.V. (1852), *Nouvelle Méthode Anatomique pour l'Investigation du Système Nerveux*, Georgi, Bonn; Waller, A.V. (1852), A new method for the study of the nervous system, *London J. Med.* **43:** 609-625.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T. (1971), The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*, *J. Am. Chem. Soc.* **93**: 2325-2327.

Weiss, P.A. (1970), Neuronal dynamics and neuroplasmic flow, pp. 840-850 in: *The Neurosciences: Second Study Program,* Schmitt, F.O. (ed.), Rockefeller University Press, New York.

Weiss, P., and Hiscoe, H.B. (1948), Experiments on the mechanism of nerve growth, *J. Exp. Zool.* **107:** 315-395.

Willard, M., Cowan, W.M., and Vagelos, P.R. (1974), The polypeptide composition of intra-axonally transported proteins: evidence for four transport velocities, *Proc. Natl. Acad. Sci. USA* **71:** 2183-2187.

Willard, M.B., and Hulebak, K.L. (1977), The intra-axonal transport of polypeptide H: Evidence for a fifth (very slow) group of transported proteins in the retinal ganglion cells of the rabbit, *Brain Res.* **136:** 289-306.

Willoughby, E.W., and Lambert, A. (1983), A sensitive silver stain for proteins in agarose gels, *Analytical Biochemistry*, **130**: 353-358.

Wilson, Leslie, ed. (1982), *Methods in Cell Biology* , Volume 24, Academic Press, Inc., New York.

Wuerker, R.B., and Kirkpatrick, J.B. (1972), Neuronal microtubules, neurofilaments, and microfilaments, *Int. Rev. Cyt.* **33:** 45-75.